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FULL RESEARCH PAPER

Application of *Pantoea agglomerans* CPA-2 in combination with heated sodium bicarbonate solutions to control the major postharvest diseases affecting citrus fruit at several mediterranean locations

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Abstract We determined the potential of using a formulated product based on *Pantoea agglomerans* CPA-2, either alone or in combination with heated sodium bicarbonate (SBC) solutions, to control the major postharvest diseases affecting citrus crops in the Mediterranean region. Treatments applied either individually or in combination were tested in semi-commercial and commercial trials carried out with oranges and mandarins from the Algarve, Andalusia and Catalonia. Firstly, several formulations of the biocontrol agents were tested in laboratory trials; one of them, a freeze-dried formulation of *P. agglomerans* strain CPA-2 called FD10-3, was chosen for combined with SBC. This formulation, applied at 2×10^8cfu ml^{-1} and the SBC treatment, applied at 3% 50°C for 20–40 s, demonstrated that it was possible to reduce decay development in laboratory trials. Semi-commercial applications of FD10-3 and 3% SBC solution at 50°C for 40 s showed excellent control of decay in unwounded mandarins and oranges artificially inoculated with both *Penicillium digitatum* and *P. italicum*. No rind injuries or residues attributable to hot water or SBC were observed on treated fruits. Combined treatment provided better control than the two treatments applied separately. Commercial trials demonstrated an important reduction in natural decay with the treatment of SBC 3% at 50°C for 40 s. Furthermore, bacterial-product formulation treatment significantly reduced the percentage of infected fruit and in some cases this reduction was equal to chemical treatments. Even so, no improvement in efficacy was observed with the combination of FD10-3 and SBC in the commercial test. We also assessed the ability of FD10-3 to grow at the wound site in oranges, whether alone or in the presence of SBC, and also its compatibility with standard citrus packinghouse practices.

Keywords Biological control · *Penicillium digitatum* · *Penicillium italicum* · Commercial trials · Baking soda · Oranges · Mandarins

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Introduction

Postharvest synthetic fungicides, such imazalil and thiabendazole, are the usual way of controlling postharvest pathogens affecting citrus fruit, including *Penicillium digitatum* and *P. italicum*. These pathogens, which respectively cause green and blue mould, constitute the most important sources of postharvest decay in citrus fruits on the world scale. They are present in all production areas with Mediterranean climates (Eckert and Eaks 1989). However, due to public demand, the development of pathogen resistance and the lack of replacement fungicides, there is major pressure to reduce or eliminate their use. Biological control, using microbial antagonists, is considered a desirable alternative to synthetic fungicides, whether applied individually or as a part of an integrated pest management policy to reduce pesticide use.

A reduced number of microbial antagonists have been registered and commercialised for postharvest use under the trade names Aspire (*Candida oleophila*) and BioSave-100 and 110 (two strains of *Pseudomonas syringae* ESC-10 and ESC-11, respectively). Fresh cells of *Pantoea agglomerans* CPA-2 which has demonstrated its efficacy in controlling green and blue mould on citrus fruits (Viñas et al. 2001) and the major postharvest diseases on apples and pears (Nunes et al. 2001, 2002) could serve as the basis for a new biocontrol product. Its ability to control multiple diseases in different hosts is an added value that makes it an interesting commercial product because, in general, biological control agents tend to have a narrower spectrum of activity than fungicides (Janisiewicz and Bors 1995).

Particularly in citrus fruit, biological control is often less effective than many commercially available fungicides and must therefore be combined with diluted fungicides (Droby et al. 1993; 1998). The use of certain exogenous substances, such as glycolchitosan, ethanol, and various different salts, has been studied to enhance the biocontrol capability of an antagonist on fungal pathogens and also as an alternative to applying chemical treatments on citrus fruits (El-Ghaouth et al. 2000; Karabulut et al. 2001; Smilanick et al. 1995; 1999). Physical treatments based on dipping fruits into hot water have also been proposed for the control of postharvest decay in citrus (Schirra et al. 2000; Palou et al. 2001). Recent studies have shown that the combination of two or more different non-fungicidal postharvest treatments could have synergistic effects and increase their efficacy in reducing the development of decay on citrus. Such treatments could, for example, combine hot water, sodium bicarbonate and biocontrol agents (Obagwu and Korsten 2003; Porat et al. 2002; Teixidó et al. 2001).

At present, one of the principal goals in the development and implementation of successful biological control products is improving the ability of antagonists to successfully control postharvest diseases under a wide variety of conditions. Along these lines, this work examines a variety of different environmental conditions such as the region in which the trials were conducted (the Algarve, Andalusia and Catalonia), different cultivars (oranges and mandarins), different pathogen strains (strains isolated from infected fruits at each location) and also the different laboratories and packinghouses used to carry out the experiments. Furthermore, all treatments were tested at all locations and during the same season. The objectives were: (i) to evaluate the effectiveness of different cell-formulations of the biocontrol agent *P. agglomerans* CPA-2, (ii) to improve the biocontrol activity of the bacterium CPA-2 by combining it with hot water and sodium bicarbonate treatments, and (iii) to test the commercial potential of treatments to be used in commercial packing lines.

Materials and methods

Fruits

Valencia late oranges and Clementina Fina mandarins from the Algarve (southern Portugal) and Valencia late oranges and Clemenules mandarins from Tarragona (Catalonia, western Spain) and from Huelva (Andalusia, southern Spain) were hand-harvested at commercial maturity (in October for mandarins and in March for oranges) in fields next to each location. Laboratory, semi-commercial and commercial trials were carried

out during three consecutive seasons: 2001 to 2002, 2002 to 2003 and 2003 to 2004, respectively.

Inoculum preparation and inoculation

Penicillium digitatum and *P. italicum* were isolated from decayed oranges from each location at which the assays were carried out (Andalusia, the Algarve and Catalonia), and were maintained on potato dextrose agar (PDA) medium. These were the most aggressive isolates of the Instituto de la Grasa (Andalucia), University of Algarve (the Algarve) and UdL-IRTA Centre (Catalonia) culture collection and were periodically transferred through citrus fruits. Conidial suspensions were prepared by adding 10 ml sterile water with 0.01% Tween 80 on 1–2 week-old cultures grown on PDA and then rubbing the surfaces with a sterile glass rod. The concentration of the conidial suspensions was determined with a haemocytometer and adjusted to 10^5 spores ml^{-1} for mandarins and 10^6 spores ml^{-1} for oranges. For laboratory trials, fruits were inoculated by wounding the flavedo on the equator of each fruit with a steel rod (1 mm wide and 2 mm long tip) that had been previously immersed in the conidial suspension of the pathogen. For semi-commercial trials, fruits without artificially induced wounds were inoculated by dipping them in a suspension of *P. digitatum* and *P. italicum* for 30 s.

Antagonist

The CPA-2 strain of *P. agglomerans* used in this study was the original antagonistic isolate, maintained in the Spanish Culture Type Collection (CECT, University of Valencia, Valencia, Spain). Stock cultures were stored at 5°C and subcultured on nutrient yeast dextrose agar (NYDA: 8 gl^{-1} nutrient broth, 5 gl^{-1} yeast extract, 10 gl^{-1} dextrose and 20 gl^{-1} agar). For laboratory and semi-commercial trials, cells were grown in 5 l sucrose-yeast medium (5 gl^{-1} yeast extract, 10 gl^{-1} sucrose) in a Biostat A fermentor (Micro-DCU 300, B.Braun Biotech International, Melsungen, Germany). The growth conditions were: 18–22 h at 30°C and 150 ml min^{-1} oxygen rate at 300 rpm, as described by Costa et al. (2001). Cells were harvested by centrifugation (6981 *g* for 10 min at 15°C) at the beginning of the stationary phase. Cell paste was resuspended in 0.05 M phosphate buffer at pH 6.5. For commercial trials, production took place in a 90 l fermentor (Ilerfred, Lleida, Catalonia), under the following growth conditions: agitation 200 rpm, at 25°C, aeration 2700 l h^{-1} and initial inoculum 10^6 cfu ml^{-1} (400 ml). Bacterial cells from the fermentor culture were harvested by centrifugation at 7520 *g* for 15 min at 10°C in an Avanti J-20 XP centrifuge (Beckman Coulter, Palo Alto, CA). Cell paste was resuspended in 0.05 M potassium phosphate at pH 6.5. Both the production and formulation processes were performed at the Postharvest Unit of the Centre UdL-IRTA and fresh cells and formulated products were sent (under cold storage conditions) to each location just one to two days before starting trials.

Efficacy of different formulations of *P. agglomerans* in oranges

Pantoea agglomerans cells were freeze-dried as described by Costa et al. (2002). Four formulated preparations consisted of freeze-dried cells with a protective 5 or 10% sucrose solution and stored in glass vials at 4°C for periods of 3 and 5 months. Formulations were coded as FD5-3 (5% sucrose with 3 months' storage at 4°C), FD5-5 (5% sucrose with 5 months' storage at 4°C), FD10-3 (10% sucrose with 3 months' storage at 4°C) and FD10-5 (10% sucrose with 5 months' storage at 4°C). In all cases the rehydration medium was non-fat skimmed milk at 1%. After wound-inoculation with pathogens (*P. digitatum* or *P. italicum* 10^6 spores ml^{-1}), 15 µl of bacterial suspension (2×10^8 cfu ml^{-1}) of each formulation was applied to the wounds. The control treatments were distilled water and fresh cells of *P. agglomerans*. The number of infected wounds was counted after 7 days at 20°C. Ten fruits constituted a single replicate and each treatment was repeated four times. The test was carried out with oranges at all locations.

Hot water and sodium bicarbonate treatments

Three stainless steel buckets, each holding 15 l of bicarbonate sodium solution (SBC), were

heated to the test temperature in a 172 l stainless steel tank fitted with a 9 kW electrical resistance heater and thermostat. The tested temperature was 20°C (control) and five higher temperatures (40, 50, 60, 65 and 70°C), two immersion periods (20 and 40 s) followed by a 5 s rinse. A solution of 3% SBC w/v was applied in all trials. Metallic grid baskets containing mandarins or oranges previously inoculated with *P. digitatum*, as described above, were submerged in the buckets. Each treatment was applied to four replicates of 20 fruits each. Treated fruits were stored at 20°C and 85% RH. The number of infected fruits was recorded after 7 days of storage and fruits were also classified into one of four categories, according to rind appearance: (i) no rind damage, (ii) slight blemishes present, (iii) moderate blemishes present, (iv) severe rind injury. These trials were carried out once for each cultivar in Catalonia.

Combination of sodium bicarbonate with *P. agglomerans* formulation cells

To determine the effectiveness of formulation FD10-3 in combination with SBC, semi-commercial trials were carried out with both oranges and mandarins in Andalusia, Algarve and Catalonia. Before treatment, fruits were dipped in a bath containing a conidial suspension of the two pathogens; *P. digitatum* and *P. italicum*, at 10^6 and 10^5 conidia ml^{-1} for oranges and mandarins, respectively. After drying, applied treatments were: (i) water for 30 s (control); (ii) fresh cells of *P. agglomerans* at 2×10^8 cfu ml^{-1} for 30 s (fresh cells); (iii) FD10-3 formulation (2×10^8 cfu ml^{-1}) for 30 s (FD10-3); (iv) 3% SBC at 50°C for 40 s followed by a 5 s rinse (SBC); (v) combination of 3% SBC at 50°C for 40 s followed by a 5 s rinse, then FD10-3 formulation (2×10^8 cfu ml^{-1}) for 30 s (SBC + FD10-3); (vi) chemical fungicide (imazalil) applied at 1250 ppm for 30 s (Iz). All treatments were applied by dipping fruits in a bath. After treatment, fruits were kept at 20°C for 24 h then stored at 5°C for 5 days. After cold storage, they were kept at 20°C for 15 days. Eighty fruits per replicate, with five replicates were used for each treatment. The number of infected fruits was then evaluated. To verify the effectiveness of FD10-3 formulation and/or the SBC solution, a trial simulating commercial conditions was conducted with 'Valencia late' oranges at the three previously cited locations. The treatments were: 3% SBC 50°C solution for 40 s, rinse for 5 s; FD10-3 2×10^8 cfu ml^{-1} for 30 s applied in a bath (except in Catalonia where FD10-3 was applied at 2×10^9 cfu ml^{-1} by spraying); SBC followed by FD10-3 treatments; Iz 1250 ppm for 30 s and water at room temperature for 30 s. Fruits were maintained for 24 h at 20°C and then cold-stored for 5 days followed by 21 days at room temperature. Each treatment was applied to three replicates of 500 fruits each.

Population dynamics of the biocontrol agent on the fruit surface

The population of *P. agglomerans* was monitored in commercial trials carried out in the Algarve and Catalonia. Fruits were wounded at four points on their surfaces and treated in baths containing either only FD10-3 at 2×10^8 cfu ml^{-1} for 30 s or a combination of FD10-3 and SBC (SBC treatment followed by FD10-3 treatment). Populations of FD10-3 alone and the presence of SBC were monitored 0, 1, 7, 15, 21 and 28 days after treatment. Each treatment was replicated six times with five oranges per treatment. Four pieces of peel, with a surface area of 2.5 cm^2 (including the wound) were removed from each orange using a cork borer. The peel surface was placed in 50 ml of 0.05 M phosphate buffer and shaken on a rotary shaker for 20 min at 150 rpm and then sonicated for 10 min in an ultrasound bath. Serial 10-fold dilutions of washings were made and plated on NYDA medium supplemented with Iz. Colonies were counted after incubation at 30°C for 24 h. Population sizes were expressed as cfu/wound.

Statistical analysis

Analyses of variance were conducted on the incidence (%) of decayed fruit using general linear model (GLM) procedure (SAS Institute Inc, Cary, NC, USA). Arcsin-square root transformation was performed before data analyses.

Statistical significance was judged at the $P < 0.05$ level and least significant difference (LSD) procedure was used to separate means. *Pantoea agglomerans* population (cfu/wound) was log-transformed to improve homogeneity.

Results

Efficacy of different formulations of *P. agglomerans* in oranges

In both fresh cells and formulations, the antagonistic bacterium CPA-2 strongly inhibited development of blue and green mould on Valencia late oranges, especially in trials carried out in the Algarve and Andalusia (Fig. 1). In fruits stored at 20°C for 7 days, significant reductions ($P < 0.05$) were obtained after biocontrol treatments against green mould in trials carried out in Andalusia (from 87% to 97%); the best results were obtained by fresh cells followed by FD10-3 and FD10-5 formulations (Fig. 1A). For blue mould, reductions were also significant ($P < 0.05$) with fresh cells, the FD10-3 formulation (from 57% to 94%) providing the best control of decay (Fig. 1B). In the Algarve, all formulations significantly reduced ($P < 0.05$) the incidence of green and blue mould in relation to control fruits (Fig 1C, D). No significant differences were observed when the different formulations were used against *P. digitatum*, the reduction achieved with these treatments being >60% (Fig. 1C). The best results against *P. italicum* were obtained with the FD10-3 formulation (reduction of 92%), while no differences were observed between the other formulations and fresh cells (Fig. 1D). Reductions of both pathogens were also observed in trials carried out in Catalonia, especially for blue mould, where FD10-3 reduced the incidence of the disease by 90%. In contrast, only a 10% of reduction was obtained for green mould (data not shown). Considering all the trials carried out, the greatest effectiveness for controlling *Penicillium* spp. decay was obtained using the formulation coded as FD10-3.

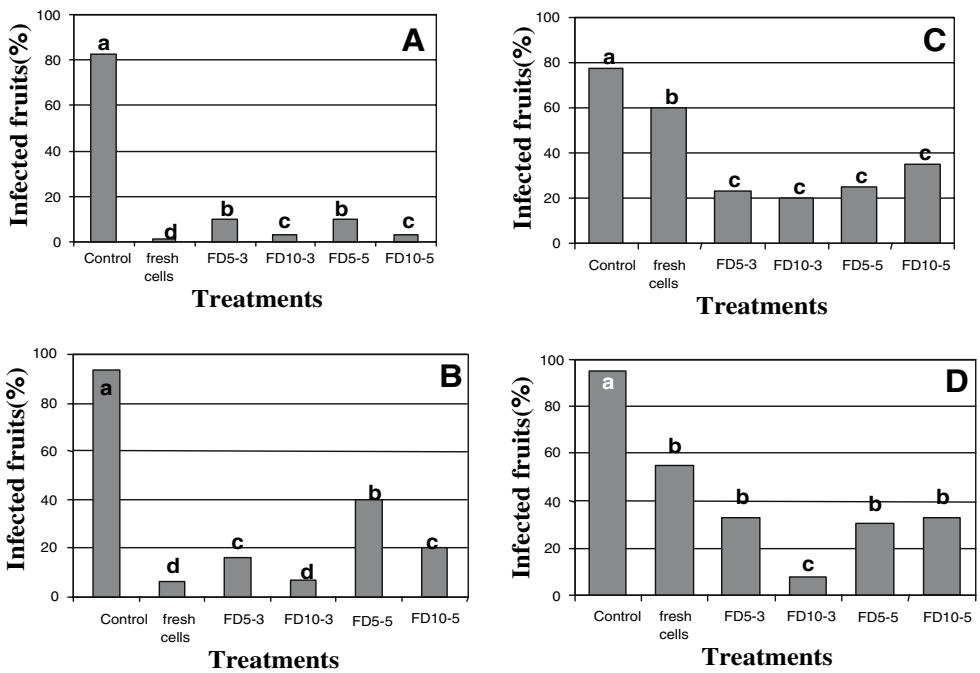

Fig. 1 Effect of several formulations (FD5-3, FD10-3, FD5-10 and FD10-5) and fresh *Pantoea agglomerans* cells CPA-2 on Valencia late oranges after previous inoculation with *Penicillium digitatum* or *P. italicum* at 10^6 spores ml^{-1} from Andalusia (**A** and **B**) and the Algarve (**C** and **D**), respectively. Fruits were kept at 20°C and 80% RH for 7 days. Columns with different letters are significantly different ($P < 0.05$) according to the LSD test

Hot water and sodium bicarbonate treatments

The combined effects of the SBC concentration, temperature and immersion period were evaluated on mandarins and oranges (Fig. 2). In oranges, SBC dips at high temperature (from 40°C to 70°C) improved green mould control with respect to hot water dips at the same temperature, for both 20 s and 40 s. This improvement was smaller in mandarins, especially for 40 s. An increase in the time of the immersion period did not significantly improve control of the incidence of green mould. In all cases, an enhancement of the effectiveness of SBC was observed for both citrus varieties when this treatment was combined with hot water as opposed to applying hot water only. The combined effect of the two treatments was greater for oranges (Fig. 2A, B). No heat damage was observed after 7 days of storage at 20°C for mandarins at 40, 50 and 60°C, or for oranges at 40, 50, 60 and 65°C (data not shown).

Combination of sodium bicarbonate with *P. agglomerans* cells formulations

In both oranges and mandarins, all the treatments tested significantly reduced decay due to both *Penicillium* strains. Applications of the biocontrol agent, as either fresh cells or formulations, significantly controlled *Penicillium* decay with respect to control fruit (Fig. 3). The major pathogen present on fruit was *P. digitatum*. In oranges from Andalusia (Fig. 3A) and mandarins from the Algarve (Fig. 3B), FD10-3 applications reduced the incidence of decay by 50%. Individually applied SBC and FD10-3 treatments produced similar results. Nevertheless, the combination of SBC with FD10-3 produced a synergistic effect, showing a higher level of decay control than either of the treatments applied individually (with a reduction by around 75% for both mandarins and oranges). In some trials, the control of decay was similar to that obtained with the imazalil treatment (Fig. 3A). In Catalonia, semi-commercial trials on oranges showed a significant reduction in decay for all treatments with respect to the control: in particular, the combination of SBC with FD10-3 reduced the incidence of decay by up to 78%. For fresh cells in mandarins, the combination of SBC with FD10-3 and chemical treatments significantly reduced the incidence of fruit decay. There were no significant differences between the control and FD10-3 and SBC treatments (data not shown).

In trials simulating commercial conditions, SBC treatments produced reductions in *Penicillium* decay of over 63% with respect to non-treated fruits (Fig. 4). The biocontrol agent produced a significant reduction in fungal decay

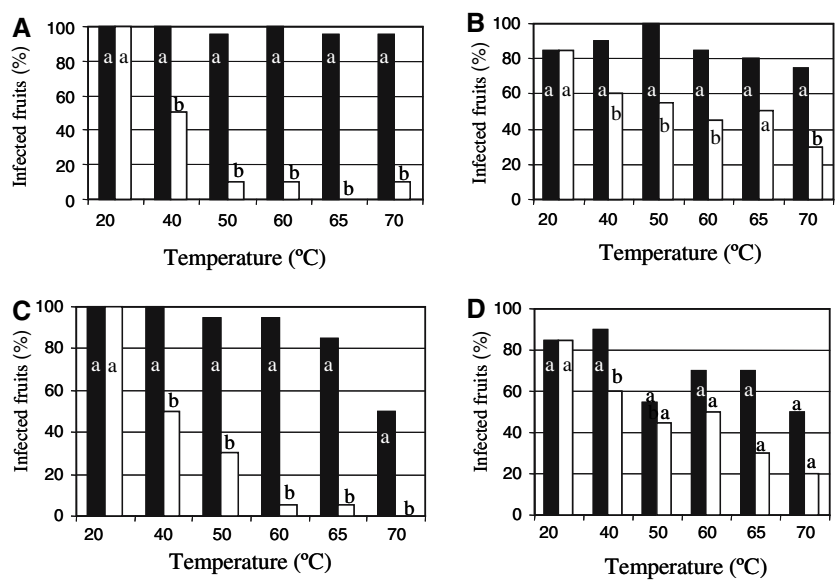

Fig. 2 Incidence of blue mould on artificially inoculated Valencia late oranges (**A** and **C**) and Clemenules mandarins (**B** and **D**) immersed for 20 s (**A**–**B**) or 40 s (**C**–**D**) in water (■) or SBC 3% (□) at different temperatures and stored at 20°C and 80% RH for 7 days. For each treatment, columns with different letters are significantly different ($P < 0.05$) according to the LSD test

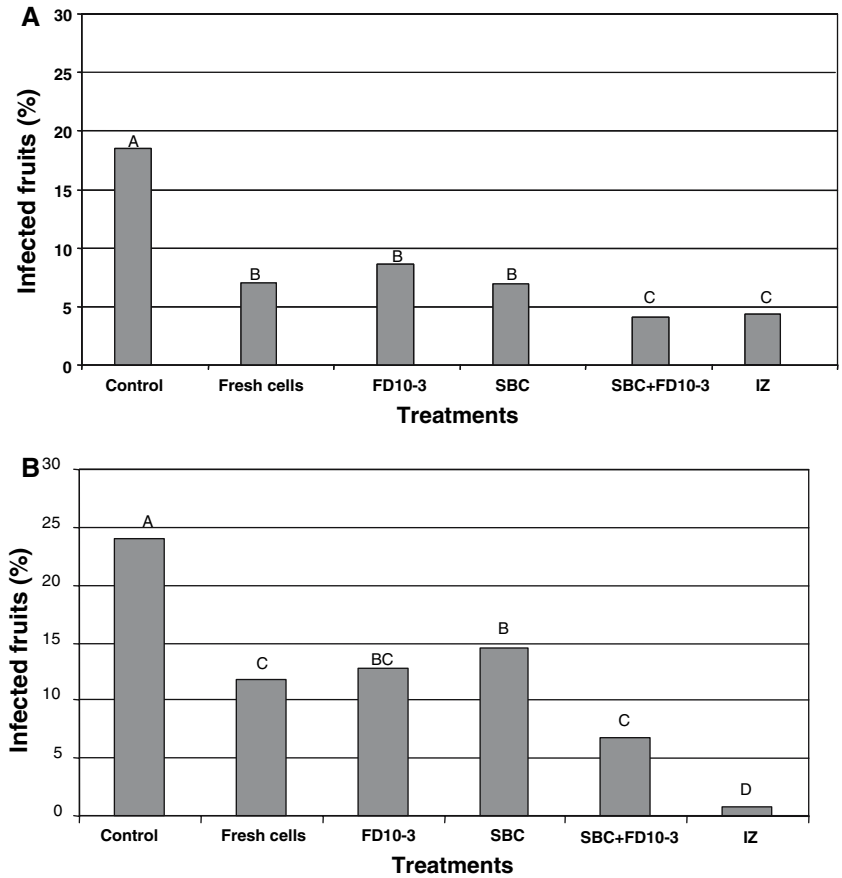

Fig. 3 Trials to evaluate the incidence of *P. digitatum* in Valencia late oranges from Andalusia (**A**) and Clementina fina mandarins from the Algarve (**B**) treated with fresh cells, formulation cells of *P. agglomerans* (FD10-3), sodium bicarbonate (SBC 3% 50°C for 40 s), and a combination of both these treatments (SBC + FD10-3) and imazalil (Iz). After treatment, fruits were kept for 24 h at 20°C and then stored for 5 days at 5°C plus 15 days at 20°C. Columns with different letters are significantly different ($P < 0.05$) according to the LSD test

(of around 40%), but this was smaller than that associated with SBC. Combining the two treatments did not produce any significant improvement with respect to the use of SBC alone. Trials in Andalusia showed a lower incidence of decay (4.1% of control) while SBC in combination with the formulation produced a more significant reduction in decay than either the control treatment or the fungicide imazalil (Fig. 4A). In the Algarve all treatments reduced the incidence of *Penicillium* decay, but there was no significant difference between treatments involving SBC and the formulation (Fig. 4B). In trials conducted in Catalonia, combining treatments resulted in excellent control over the most important postharvest diseases, with better results than that obtained by chemical treatment (with 80% and 40% reductions in decay, respectively). The higher incidence in Andalusia and the Algarve was caused by *P. digitatum*, while in Catalonia was due to *P. italicum*.

Population dynamics of the biocontrol agent on the fruit surface

The study of population dynamics in commercial trials involving wounded fruits showed differences between fruits treated only with cell formulations and those treated in combination with SBC in the Algarve and Catalonia. In trials carried out at the Algarve, at time zero the population recovered from both FD10-3 alone and FD10-3 applied in combination with SBC was around 1.5×10^5 cfu/wound (Fig. 5). After 24 h at 20°C, the population greatly increased in both cases and then remained stable during 21 days of cold and room storage. After that, a slight decrease was observed. At the end of the experiment, the populations recovered were respectively 7.0×10^5 cfu/wound and 6.5×10^6 for fruit treated only with cell formulations and combined with SBC. A similar pattern was observed in Catalonia (data not shown).

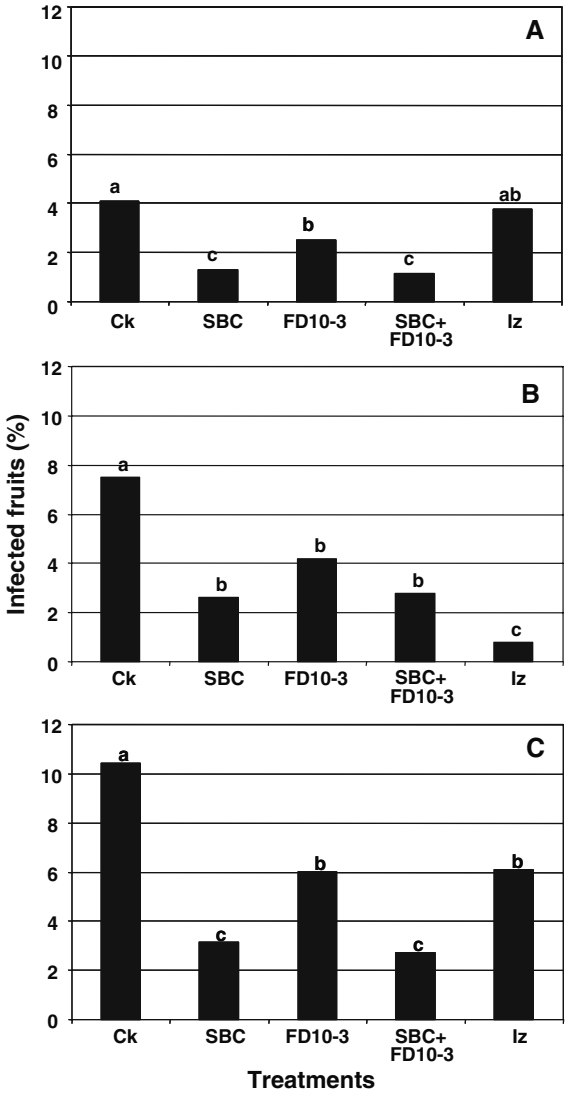

Fig. 4 Trials in unwounded and naturally infected Valencia late oranges from (**A**) Andalusia, (**B**): the Algarve and (**C**) Catalonia. Fruits were treated with sodium bicarbonate (SBC 3% 50°C for 40 s), formulation cells of *P. agglomerans* (FD10-3), and a combination of both these treatments (SB + FD10-3) and imazalil (Iz). After treatment, fruits were stored for 24 h at 20°C and then kept for 5 days in cold storage followed by a further 15 days (Andalusia and Catalonia) and 21 days (the Algarve) at 20°C

Discussion

In general, the effectiveness of biocontrol agents, hot water and SBC applied alone rarely reached that obtained with fungicides. This work suggests an alternative to chemical control involving the

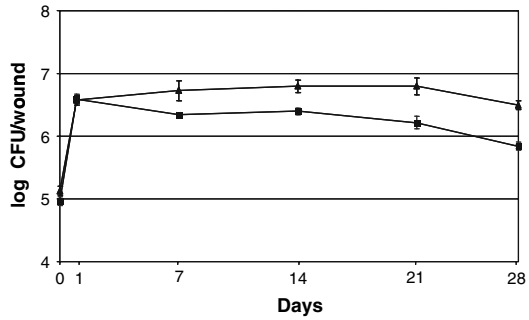

Fig. 5 Population dynamics of cell formulations of *P. agglomerans* (FD10-3 ■) in wounded 'Valencia late' oranges from the Algarve, both alone and in the presence of SBC (▲). Fruits were wounded and treated as in commercial trials and maintained for 24 h at 20°C and stored for 5 days at 5°C followed by 21 days at 20°C. Vertical bars indicate mean standard error

combination of a formulation of *P. agglomerans* CPA-2 with a hot water solution of SBC, which could be implemented in packinghouses to control the most important postharvest diseases affecting citrus fruits, with a similar level of efficacy as chemical treatments. The goal of this study was further expanded to evaluate alternatives to chemical treatments at different locations in the Mediterranean area. The aim was to cover as wide a range of conditions as possible, with different pathogen strains and fruits from different soils and fields using a bacterium-formulated product, developed according to a scale up programme with regard to both production and formulation processes (Costa et al. 2001; 2002).

In order to use a biocontrol-based product on a commercial scale, it is necessary to develop a shelf-stable formulated product that retains similar biocontrol activity to that of fresh cells (Janisiewicz and Jeffers 1997). Our research showed that, in general, the effectiveness of all the formulations tested was similar to or greater than fresh cells, although treatment with FD10-3 tended to produce a greater degree of control than the others. The efficacy of FD10-3, a freeze-dried cell-based product, against *P. digitatum* in oranges had been previously evaluated in laboratory trials (Costa et al. 2002). The present work, which was carried out with oranges from different geographical locations and with different pathogen strains, confirmed and reproduced the previously obtained results. Several authors have

described the efficacy of fresh yeast/bacteria cells for controlling postharvest decay in citrus fruits (Arras 1993; Chalutz and Wilson 1990; Droby et al. 1993; McGuire 1994; Porat et al. 2002; Smilanick and Denis-Arrue 1992). However, very little information was previously available relating to the efficacy of biocontrol agent formulations. Only Droby et al. (1998) evaluated the efficacy of Aspire, a *C. oleophila*-based product, in controlling postharvest decay in citrus fruit in commercial packinghouse tests.

The beneficial effects of hot water treatments for controlling *Penicillium* rot in citrus have been previously reported (Houck 1967; Palou et al. 2001; Schirra and D'hallewin 1997; Smilanick et al. 1995). In these studies, the temperature required to control green mould was between 30°C and 55°C, with a relatively long immersion period (1–5 min). However, immersion at temperatures above 53°C was reported to produce phytotoxic effects. Different strategies for applying hot water at higher temperatures have been satisfactorily studied in an attempt to reduce the contact time between fruit and water. These include hot water brushing (Porat et al. 2000) and drench treatment (Smilanick et al. 2003). The present work shows that the application of hot water dips at temperatures of between 40°C and 60°C for shorter exposure periods (20 and 40 s) did not injure fruit, but was insufficient to significantly reduce green mould decay. Similar results were found by Palou et al. (2002) who applied hot water at 45°C and 50°C for 150 s to control green and blue postharvest moulds on Clementine mandarins.

It has been demonstrated that heated chemical solutions were capable of improving the control of both green and blue mould in comparison with applying only a hot water treatment (Barkai-Golan and Philipps 1991; Lurie 1999; Palou et al. 2001, 2002; Smilanick et al. 1997). Our work supports this finding, because hot SBC solutions at 3% significantly reduced green mould at all the temperatures tested (from 40 to 70°C) and for both of the immersion periods studied (20 and 40 s). The efficacy of the treatments was greater in oranges than in mandarins. Although a high degree of efficacy was obtained at all of the temperatures tested, rind injuries were observed at over 60°C, in both oranges and mandarins. No bicarbonate residue was observed on the fruit, which suggested that the water rinsing applied in our studies was sufficient to maintain the external appearance of the fruit without reducing the effectiveness of the treatment.

Given the relatively reduced inhibitory activity of SBC, subsequent treatments could be needed to provide protection from re-infection. The effectiveness of applying SBC and sodium carbonate was significantly improved when these treatments were followed by a biological control treatment with *P. syringae* strain ESC10 (BioSave 100) applied at 10^9 cfu ml^{-1} or with Iz at 1,000 μg ml^{-1} (Smilanick et al. 1999). However, for these complementary treatments to be used reliably under commercial conditions, it is necessary to previously conduct a study to assess the tolerance of the biocontrol agent to the chemical solutions involved. Teixidó et al. (2001) demonstrated that *P. agglomerans* CPA-2 is totally tolerant and compatible with SBC at 2%, but is incompatible with sodium carbonate solutions. Furthermore, growth patterns of this bacterium on artificially wounded and unwounded oranges were similar, whether applied individually or in combination with SBC. Although these experiments showed the potential of using this biocontrol agent alone or in combination with SBC, semi-commercial and full-scale commercial evaluation was necessary to demonstrate its potential applicability in the citrus industry.

Semi-commercial trials carried out in this work showed an excellent control of decay in mandarins and oranges stored under commercial conditions with the combination of FD10-3 and sodium bicarbonate 3% at 50°C for 40 s. No rind injuries were observed in our work; therefore dip treatments at this temperature for short-immersion periods apparently removed the risk of injury and proved sufficient to control the most important pathogens present in the Mediterranean area. In practically all assays, the combined treatment provided a better degree of control than either of the treatments applied separately. Although SBC applied alone provided a great degree of control over decay, the results of this treatment were often highly inconsistent, whereas the combined treatment always provided an excellent level of

control. The benefits of using biocontrol agents, heat and SBC are well documented in our semi-commercial trials. Porat et al. (2002) also showed the excellent level of control of *P. digitatum* after the treatment with hot water brushing at 62°C for 20 s and SBC 2% followed by the application of *C. oleophila* in semi-commercial postharvest trials with oranges and grapefruit.

Our commercial results demonstrated that the use of hot water plus SBC may be applied in citrus fruit packinghouses because this combination showed an important reduction in natural decay, mainly due to both *P. digitatum* and *P. italicum*. Furthermore, the treatment involving only the bacterium-product formulation FD10-3 significantly reduced (by more than 40%) the percentage of infected fruits in relation to the control and in some cases this reduction was equal to that obtained by applying chemical treatment. In spite of our positive results in semi-commercial trials, no improvement in efficacy was observed with the combination of FD10-3 and SBC in commercial tests. Probably, when a low incidence of re-infection appeared during postharvest management the protection effect of the biocontrol agent was not apparent in the combined treatment.

The ability of FD10-3 to grow in wounds on oranges either alone or in the presence of SBC was assessed in commercial trials. Maintaining oranges at 20°C for 24 h before cold storage increased the population of the biocontrol agent by more than 10-fold, which suggests that *P. agglomerans* could colonize wounds. However, it should be pointed out that during this same period both *P. digitatum* and *P. italicum* showed 100% germinated spores (Plaza et al. 2003). In previous work, Teixidó et al (2001) also reported the excellent adaptation of this strain to cold storage temperatures and its survival in wound sites. Furthermore, the same study demonstrated that *P. agglomerans* had a population growth dynamic that was similar on wounded and unwounded oranges, but population levels were always lower on unwounded oranges. A low population and an irregular distribution of the antagonist on intact surfaces could reduce the spatial coincidence with the pathogen-wound and, therefore, the efficacy of the biocontrol agent.

To implement the combined proposal at the commercial scale, more effort should be put into improving the FD10-3 formulation in order to increase the dispersion and adherence of this product, and to promote a greater understanding of interactions between this antagonist, citrus pathogens and environmental factors. This should help to improve the commercial application of this potential biological product. Application of CPA-2 should be compatible with existing citrus packinghouse practices and its use would imply minimal environmental and worker safety concerns in comparison with chemical applications. Application of hot water and SBC could offer another safe postharvest treatment and an alternative to commercial treatment with imazalil. This option would also be compatible with normal practice in many citrus packinghouses in the Mediterranean area.

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