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Improving control of green and blue molds of oranges by combining *Pantoea agglomerans* (CPA-2) and sodium bicarbonate

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Abstract

The potential of using *Pantoea agglomerans* (strain CPA-2) alone, or in combination with sodium bicarbonate or sodium carbonate solutions, for control of *Penicillium digitatum* (green mold) and *Penicillium italicum* (blue mold) on oranges was investigated under ambient (20 °C) and cold storage (3 °C) conditions. *P. agglomerans* controlled both pathogens on oranges at 2×10^8 cfu ml^{-1}. The biocontrol agent was found to be completely tolerant to 2% sodium bicarbonate at room temperature, although its culturability was reduced by >1000-fold after 30 min in 2% sodium carbonate. The efficacy of *P. agglomerans* for control of green mold was improved when combined with sodium bicarbonate, resulting in complete and 97.6% reduction of decay incidence at 3 °C and 20 °C, when compared to untreated controls. Satisfactory results were also obtained with the combined treatment for control of blue mold. *P. agglomerans* grew well inside wounds on oranges at both 20 °C and 3 °C. In contrast, it showed a reduced growth on the surface of intact fruit. Sodium bicarbonate at 2% concentration did not noticeably affect antagonist population development. Thus, use of bicarbonate treatment at 2% followed by the antagonist *P. agglomerans* CPA-2 could be an alternative to chemicals for control of postharvest diseases on oranges.

Introduction

Post-harvest green and blue molds of citrus, caused by *Penicillium digitatum* (Pers.:Fr.) Sacc. and *Penicillium italicum* Wehmer, are responsible for severe economic losses worldwide (Bancroft et al., 1984; Eckert and Eaks, 1989). Currently, these post-harvest diseases are controlled by chemicals such as ortho-phenil phenate, imazalil and thiabendazol. The use of chemicals is becoming increasingly restricted because of concerns about environment and health as well as the development of resistance to these fungicides among fungal pathogens (Díaz and Vila, 1988; Eckert, 1990; Bus et al., 1991; Eckert et al., 1994). Therefore, there is a need to develop new and effective methods to control post-harvest diseases that pose no harm to human health and the environment, and are accepted as safe by the general public.

Biological control using microbial antagonists has received a great deal of attention as a promising alternative to chemicals. Many organisms, such as *Pseudomonas* spp. (Huang et al., 1991; 1993; 1995; Smilanick and Denis-Arrue, 1992), *Bacillus* spp., (Singh and Deverall, 1984; Arras and D'hallewin, 1994), *Debaryomyces hansenii* (Chalutz and Wilson, 1990), *Pichia guilliermondii* (Droby et al., 1993) and *Trichoderma viride* (De Matos, 1983) have been isolated to protect wounds from post-harvest pathogens on citrus fruits. One yeast and two bacterial products are currently commercially available in USA. *Candida oleophila* strain I-182 was registered during 1995 (US-EPA registration no 55638-29) as ASPIRE™ by Ecogen Inc. (Langhorne, PA) for the biological control of post-harvest diseases of pome and citrus fruits (Chand-Goyal et al., 1998). At the same time *Pseudomonas syringae* strains ESC-10 and ESC-11

were registered (US-EPA registration no 64296-7) as BIOSAVE-10™ and BIOSAVE-11™ by EcoScience Corp., (Orlando), FL (Bull et al., 1997) for the same purpose.

Recent studies at the University of Lleida, Catalonia, Spain have demonstrated that the strain CPA-2 of *Pantoea agglomerans*, previously classified as *Erwinia herbicola*, isolated from the apple surface, is an effective antagonist to the major fungal pathogens of apples and pears (Viñas et al. 1999). Generally, biological control agents have a relatively narrow spectrum of activity compared to fungicides (Janisiewicz and Bors, 1995). It is thus particularly important to find antagonists with a broader spectrum in terms of both hosts and pathogen control range. Recently, interest has been given to potential of combining microbial biocontrol agents with other chemical components for enhancing control of post-harvest diseases of pome, stone and citrus fruits.

Carbonic acid salts are common food additives allowed with no restrictions for many applications by European and North American regulations (Lindsay, 1985; Multon, 1988). The antimicrobial activity of these chemicals has been described *in vitro* (Marloth, 1931; Corral et al., 1988) and in a wide range of substrates as well. Sodium bicarbonate and sodium carbonate reduce the incidence of postharvest decay on citrus fruits (Barger, 1928; Fawcett, 1936; Houck, 1965; Klotz, 1973; Smilanick et al., 1997). The inhibitory activity of carbonate and bicarbonate solutions against *Penicillium* spp. is low and generally only fungistatic (Marloth, 1931; Hwang and Klotz, 1938).

Sodium carbonate and bicarbonate have disposal issues that have to be taken into account due to the large amount of sodium and the high pH of these solutions. Depending on the kind of soil and the pH of water, some problems could appear. However, sodium bicarbonate offers a real advantage to sodium carbonate. An equivalent-weight solution of sodium bicarbonate has a lower pH and less sodium than a similar solution of sodium carbonate. Equimolar amounts of sodium bicarbonate contain 27.4% sodium compared to 43.4% sodium in sodium carbonate (Smilanick et al., 1999).

In the near future, the preferred alternative to chemical treatments will be probably a combination of different methods. In fact, Smilanick et al. (1999) found that the effectiveness of sodium bicarbonate and carbonate was significantly improved when these treatments were followed by the fungicide imazalil or *P. syringae* ESC-10. The combination of bicarbonate or carbonate with *P. syringae* overcame significant shortcomings of either of these treatments alone.

This study reports: (a) the efficacy of *P. agglomerans* (strain CPA-2) for the control of *P. digitatum* and *P. italicum* rots on oranges; (b) the potential for improving the efficacy by combination with sodium bicarbonate or carbonate treatments when stored under ambient conditions (20 °C) or in cold storage (3 °C), and (c) determination of the population dynamics of the biocontrol agent in wounded and non-wounded fruits.

Materials and methods

Fruits

Valencia oranges were grown in the Baix Ebre and Montsià areas in Tarragona (Catalonia, Spain) following standard cultural practices. Fruits were selected from field bins after harvest before any commercial post-harvest treatments were applied. Oranges were used following harvest or after a short storage period at 3 °C (no longer than 2 weeks). Before each experiment the oranges were dipped in a NaOCl 0.5% solution for 1 min, rinsed with water, allowed to air-dry at room temperature and randomized.

Pathogens

P. digitatum PDM-1 and *P. italicum* PIM-1 were isolated from decayed oranges and maintained on potato dextrose agar medium (PDA; 200 ml of extract from boiled potatoes, 20 g of dextrose, 20 g of agar and 800 ml of water). These are the most virulent isolates of the University of Lleida-IRTA culture collection and to maintain virulence, they were periodically grown on wounded citrus fruits and reisolated. A conidial suspension was prepared by adding 10 ml of sterile water with 0.01% of tween 80 over the surface of 10-day-old cultures grown on PDA and rubbing the surface with a sterile glass rod. The cells were counted in a haemocytometer and diluted to a concentration of 10^5 or 10^6 spores ml^{-1}. This is a recommended concentration for the evaluation of postharvest treatments to control green and blue molds (Eckert and Brown, 1986a). In all the experiments each pathogen was tested separately.

Antagonist

P. agglomerans strain CPA-2 was obtained from the UdL-IRTA Centre, Catalonia, Spain. It was originally

isolated from an apple surface (cv. Golden Delicious). Stock cultures were stored at 5 °C and were subcultured on nutrient yeast dextrose agar (NYDA; 8 g of nutrient broth, 5 g of yeast extract, 10 g of dextrose, 20 g of agar and 1000 ml of water). For long-term storage, CRIOBILLES AEB 400100 (AES Laboratory, Combourg, France) at −80 °C were used. Bacterial suspensions for efficacy and population assays were prepared from bacteria grown in a 6-l bench-top fermenter at 30 °C in NYDB medium (NYDA medium without agar). Cells were harvested at the begining of the stationary phase (24 h) by centrifugation at 6981 *g* for 10 min. The cell paste was resuspended in 0.05 M phosphate buffer to the desired concentration.

Biocontrol of blue-mold and green-mold with P. agglomerans

Surface-sterilized oranges were wounded with a steel scalpel by making an injury 2 mm deep by 1 mm wide by 5 mm long on the equator of each fruit. The shallow wounds penetrated the albedo tissue but not the juice sacs to simulate natural infection. A 25 µl aqueous suspension of *P. digitatum* or *P. italicum* at 1×10^5 or 1×10^6 spores ml^{-1}, was applied to each wound, followed by inoculation with 20 µl of the appropriate concentration of a suspension of *P. agglomerans* CPA-2. The antagonist concentrations were adjusted to 4×10^7 and 2×10^8 cfu ml^{-1} according to a standard curve obtained spectrophotometrically by measuring transmittance at 420 nm (CECIL CE 1020). Five oranges constituted a single replicate, each treatment was repeated four times and the experiment was carried out twice. Treated oranges were incubated at 20 °C and 90% relative humidity (RH). Data were recorded as the percentage of decayed fruits after 7 days incubation.

Compatibility of P. agglomerans *with sodium bicarbonate and carbonate solutions*

One milliliter of sodium bicarbonate or sodium carbonate solutions at concentrations ten times the required, was transferred to glass test tubes with 9 ml of *P. agglomerans* at a known concentration (10^7 cfu ml^{-1}). The final required concentration for the sodium salts was 2%. After incubation for 30 min at 25 °C, suspensions ten-fold were diluted at few times in 0.05 M phosphate buffer (pH 7) and plated on Petri dishes with NYDA medium. A control with bacterial cells in water was also tested. The plates were incubated at 25 °C in the dark for 24 h and colonies were counted. The test was repeated twice.

Combining P. agglomerans *and sodium bicarbonate*

Four treatments and two different storage conditions were tested in this set of experiments. The treatments applied to pathogen inoculated fruits were: (1) control (water); (2) *P. agglomerans* CPA-2; (3) sodium bicarbonate; and (4) sodium bicarbonate + *P. agglomerans* CPA-2. Treated fruits were stored for 14 days at 20 °C and 90% RH or 60 days at 3 °C and 98% RH (long-term cold storage).

Valencia oranges were wounded and inoculated with *P. digitatum* or *P. italicum* at 10^6 spores ml^{-1}, about 2 h before treatments were applied. The control treatment consisted of dipping pathogen inoculated oranges in water for 1 min. Antagonist treatment was applied by dipping oranges for 1 min in an aqueous solution containing 2×10^8 cfu ml^{-1} of *P. agglomerans*. Based on the results of previous research with sodium bicarbonate against green (Smilanick et al., 1999; Palou et al., 1999) and blue (Palou et al., 2000) molds, a concentration of 2% sodium bicarbonate and a 150-s immersion period were chosen for the sodium bicarbonate treatment. Finally, bicarbonate and antagonist treatment were combined by first dipping fruits in 2% sodium bicarbonate for 150 s and, after allowing to air-dry at room temperature, by dipping fruit in 2×10^8 cfu ml^{-1} *P. agglomerans* suspension for 1 min. For each storage condition, each treatment was applied to three replicates of 20 oranges each. Fruits were stored in trays. Decayed fruits were recorded after 7 and 14 days storage at 20 °C and after 30 and 60 days of cold storage.

Population dynamics on the orange surface

The populations of *P. agglomerans* CPA-2 were monitored on wounded and unwounded oranges. Wounded and unwounded fruits were treated with *P. agglomerans* or sodium bicarbonate followed by *P. agglomerans*, as described in efficacy trials. Fruits were incubated at 20 °C and 90% RH, and in long-term cold storage at 3 °C and 98% RH. Bacterial populations were monitored after 0, 1, 2, 3, 7, 10 and 15 days on fruits stored at 20 °C, and after 3, 7, 15, 30, 45 and 60 days on fruit in cold storage. Four oranges constituted each

replicate and twenty five pieces of peel surface of 2.5 cm^2 from each orange were removed with a cork borer (100 pieces of peel per replicate). In the case of wounded fruits, wounds were included in one of the removed segments of peel. Peel surface segments were shaken in 100 ml sterile phosphate buffer (pH 7) on a rotatory shaker for 20 min at 150 rpm and then sonicated for 10 min in an ultrasound bath. This final step was used to improve detachment of the antagonist from the orange surface.

Serial 10-fold dilutions of the washings were made with 0.05 M phosphate buffer and plated on NYDA medium. After incubation at 25 °C in the dark for 24 h, the colonies were counted and their number per cm^2 of fruit surface were calculated for each sample. There were four replicates per treatment.

Statistical analysis

Effects of treatments on the incidence of green and blue molds were analyzed using an analysis of variance on data transformed to the arcsine of the square root of the proportion of infected fruits. This transformation was used to improve homogeneity of variances. Statistical significance was judged at the level $p < 0.05$ and least significant difference (LSD) procedure used for means separation. No statistical differences were found between experimental replications; therefore the results from both different experiments were pooled.

P. agglomerans populations on orange surfaces (cfu/cm^2) were log transformed to improve homogeneity of variances (Parbery et al., 1981) and plotted in figures where standard errors are shown for each sampling date.

Results

Biocontrol with P. agglomerans

The antagonistic bacteria, *P. agglomerans* CPA-2 strongly inhibited development of *P. italicum* and *P. digitatum* on wounded oranges artificially inoculated at both concentrations tested (Figure 1). The efficacy of 4×10^7 and 2×10^8 cfu ml^{-1} of *P. agglomerans* was not statistically different when the concentration of pathogens used was 10^5 cfu ml^{-1}, with percentages of infected wound reductions >70% and >85% for green and blue mold respectively.

When antagonistic biocontrol concentrations were increased from 4×10^7 to 2×10^8 cfu ml^{-1}, the

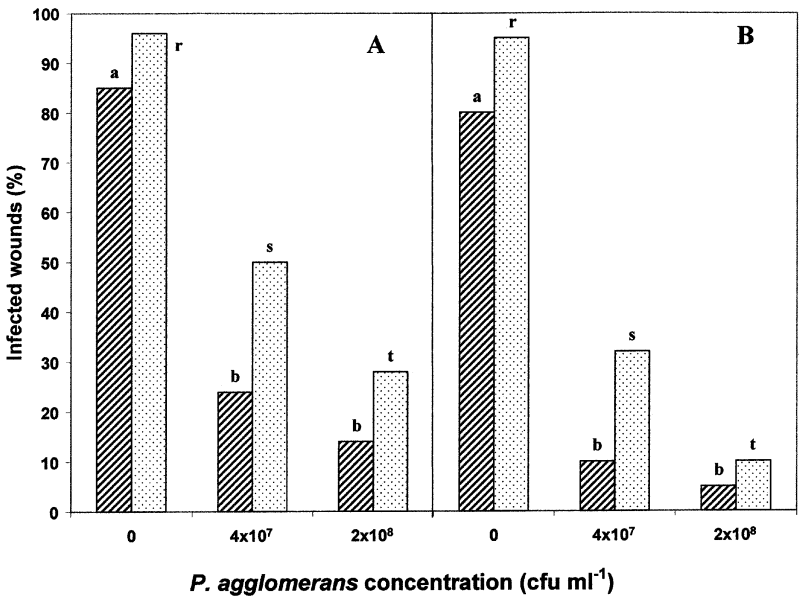

Figure 1. Incidence of green mold (A) and blue mold (B) on wounded Valencia late oranges inoculated with 10^5 (▨) and 10^6 (▩) spores ml^{-1} of *P. digitatum* or *P. italicum* respectively, followed by treatment with different concentrations of *P. agglomerans* after 7 days incubation at 20 °C and 90% RH. Within pathogen concentrations, columns with the same letter are not significantly different ($p < 0.05$) according to the LSD.

efficacy against both pathogens at 10^6 cfu ml^{-1}was significantly increased achieving reductions in incidences of *P. digitatum* and *P. italicum* of about 71% and 89% respectively.

Compatibility of P. agglomerans *with sodium bicarbonate and carbonate solutions*

The density of culturable *P. agglomerans* was not reduced after 30 min immersion in a 2% sodium bicarbonate solution compared to the control (water) (Table 1). However, the density of culturable *P. agglomerans* was reduced more than 1000-fold by 2% sodium carbonate solution.

Table 1. Effect of the immersion in sodium bicarbonate and carbonate solutions for 30 min on the density of culturable *P. agglomerans* CPA-2.

Solution	cfu ml^{-1}	Standard error
Water	9.15×10^6	1.7×10^5
Sodium bicarbonate 2%	9.9×10^6	5.7×10^5
Sodium carbonate 2%	$<1 \times 10^4$	—

cfu; colony forming units.
Limit of detection = 1×10^4 cfu ml^{-1}.
(—); Not possible to calculate Standard Error.

Enhancement of biocontrol by P. agglomerans *with sodium bicarbonate*

At 20 °C, all the assayed treatments (antagonist CPA-2, sodium bicarbonate solution and the combination) significantly decreased decay incidence either on fruits inoculated with *P. digitatum* or *P. italicum*, after 7 or 14 days incubation (Figure 2). After 7 days at 20 °C, no significant differences were found on green mold incidence on oranges treated with *P. agglomerans* and sodium bicarbonate separately (30% of roted fruits). This incidence represents a decay reduction of 57%. Efficacy was significantly improved when the treatments were combined resulting in 97.6% of decay reduction (1.7% decayed fruits). Similar results were obtained after 14 days incubation. No significant differences among treatments were found for the incidence of blue mold after 7 days incubation and all treatments reduced decay by more than 57% (38.3%, 26.7% and 21.7% decay incidence for the antagonist, sodium bicarbonate and the combination treatments, respectively).

Under cold storage conditions (3 °C) the incidence of *P. digitatum* was lower than that of *P. italicum*, with rot incidences of 18% and 52% after 30 and 60 days, (Figure 3). All treatments significantly

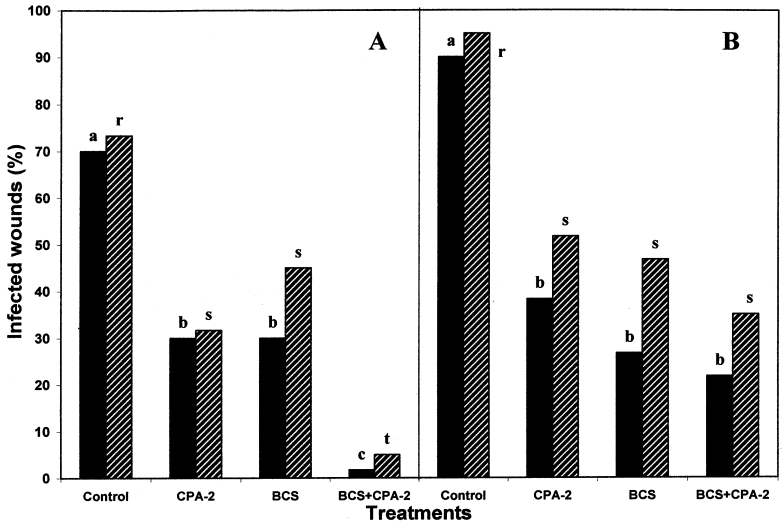

Figure 2. Incidence of green mold (A) and blue mold (B) on wounded Valencia late oranges inoculated with 10^6 spores ml^{-1} of *P. digitatum* or *P. italicum* respectively, followed by treatment with water (control prove), *P. agglomerans* (CPA-2), sodium bicarbonate (BCS) and the combination of sodium bicarbonate and *P. agglomerans* (BCS + CPA-2), after 7 (■) and 14 (▨) days incubation at 20 °C and 90% RH. Within times of incubation, columns with the same letter are not significantly different ($p < 0.05$) according to the LSD.

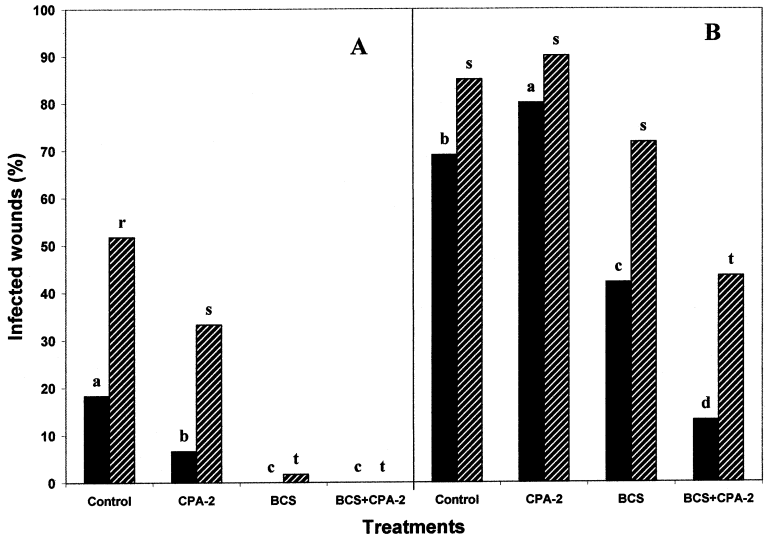

Figure 3. Incidence of green mold (A) and blue mold (B) on wounded Valencia late oranges inoculated with 10^6 spores ml^{-1} of *P. digitatum* or *P. italicum* respectively, followed by treatment with water (control prove), *P. agglomerans* (CPA-2), sodium bicarbonate (BCS) and the combination of sodium bicarbonate and *P. agglomerans* (BCS + CPA-2), after 30 (■) and 60 (▨) days incubation at 3 °C and 98% RH. Within times of incubation, columns with the same letter are not significantly different ($p < 0.05$) according to the LSD.

inhibited *P. digitatum* decay. However, the best treatments were sodium bicarbonate alone, and in combination with the antagonist, where complete (100%) control was achieved after 30 days in cold storage. *P. agglomerans* CPA-2 did not control *P. italicum* under cold storage conditions. The best results were obtained with the combination of sodium bicarbonate and *P. agglomerans*, that controlled blue mold significantly better than each treatment alone, after 30 and 60 days at 3 °C. This combined treatment showed a decay incidence of 13% after 30 days in cold storage conditions, that represents a reduction of 81% of blue mold when compared with untreated control.

Population dynamics on the orange surface

Initial populations of *P. agglomerans* CPA-2 on wounded oranges were larger than on unwounded ones in 20 °C and 3 °C storage conditions (Figure 4). The patterns of growth of *P. agglomerans* were similar, whether applied alone or in combination with sodium bicarbonate.

P. agglomerans populations increased approximately 18-fold on the surface of wounded oranges stored at 20 °C during the first 24 h. After this the population remained stable until the end of the assay (15 days). These results were identical for the antagonist treatment alone, or when combined with sodium bicarbonate. The pattern on unwounded fruits at 20 °C was very similar, but the population levels were lower.

On wounded and unwounded fruits stored at 3 °C, populations decreased approximately 4-fold during the first three days. Then, *P. agglomerans* populations strongly increased on wounded fruits and reached a maximum after 45 days of storage. In contrast, on unwounded oranges population increased slower than wounded ones after the first three days and remained stable until the end of the experiment.

Discussion

This study has demonstrated that *P. agglomerans* CPA-2 which is an effective antagonist to the major postharvest pathogens of pome fruits (Viñas et al., 1999) could also be used on oranges to control *P. digitatum* and *P. italicum*.

Efficacy trials with *P. agglomerans* CPA-2 on artificially wounded and inoculated oranges showed good biocontrol potential against both green and blue molds. The concentration of the antagonist needed to obtain satisfactory disease control (2×10^8 cfu ml^{-1}) was lower than that recommended for the commercial available

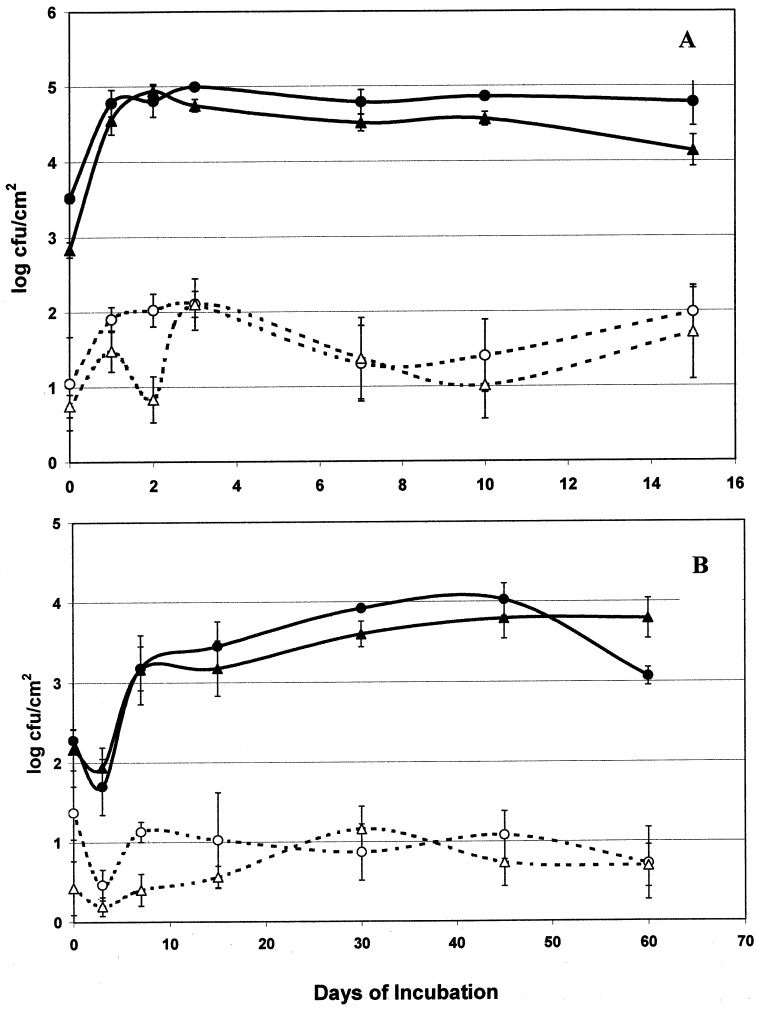

Figure 4. Population dynamics of *P. agglomerans* CPA-2 on wounded (closed symbols) and unwounded (open symbols) oranges treated with *P. agglomerans* CPA-2 (●;○) or the combination of sodium bicarbonate and *P. agglomerans* CPA-2 (▲; △) and incubated at 20 °C and 90% RH (A) and 3 °C and 98% RH (B). Points represent the means of four replications and vertical bars indicate standard errors of the means. Limit of detection: 4 cfu cm^{-2}.

product BIOSAVE, and consequently low enough to be considered viable for commercial use.

The exact mechanism by which *P. agglomerans* CPA-2 reduces decay is not clear. It has been reported that *E. herbicola*, at present classified as *P. agglomerans*, inhibits other plant pathogens by producing acid conditions (Riggle and Klos, 1972; Beer et al., 1984), causing pre-emptive colonization (Wilson et al., 1992; Kearns and Hale, 1996), inducing plant defence (Slade and Tiffin, 1984), parasitising the pathogen (Brik et al., 1998), competing by nutrients (Goodman, 1967), and producing herbicolin (Ishimaru et al., 1988) or antibiotics (Vanneste et al., 1992; Kearns and Hale, 1996). In associated studies (C. Nunes unpublished data), killed cells of *P. agglomerans* CPA-2 and its culture filtrate had no antagonistic activity against artificially inoculated pathogens. This would suggest that antibiosis is not a mechanism of biocontrol of this strain.

Biocontrol agents are poor eradicants of pathogens on citrus and are usually incapable of controlling green mold when fruits are inoculated at least 24 h before treatment (Smilanick and Denis-Arrue, 1992). Control of green and blue molds after inoculation is important because most infections occur through

wounds inflicted during or just after harvest (Powell, 1908; Green, 1932; Fawcett, 1936).

Recent work showed that sodium carbonate and sodium bicarbonate solutions, are as effective as common synthetic fungicides to control green mold on lemons (Smilanick et al., 1995) and oranges (Smilanick et al., 1997, 1999). Similar effectiveness was reported against blue mold on oranges (Palou et al., 2000). However, carbonates are poor eradicants and do not kill spores (Marloth, 1931). Their inhibitory action is not very persistent, and they do not provide protection of the fruit from re-infection after treatment. Biocontrol antagonists, which residues can persist for long periods, may accomplish this task (Smilanick et al., 1999). A combination between our antagonist and these mentioned carbonate treatments could be a reliable solution to control postharvest diseases on citrus.

P. agglomerans CPA-2 is totally tolerant and compatible with sodium bicarbonate solution at 2%. This contrasts with the incompatibility with sodium carbonate solution. Differences of pH (pH 8.3 to 8.6 for sodium bicarbonate and 11.3 to 11.5 for sodium carbonate) could explain the different effect of these solutions on the antagonist.

Excellent control of green mold after storage at 3 °C and 20 °C, was obtained with the combination of treatments. Furthermore, at 20 °C, the combination of antagonist with sodium bicarbonate provided significantly better green mold control than these treatments applied separately.

Blue mold incidence after long-term cold storage (3 °C and 98% RH) was higher than green mold incidence. These results are in agreement with earlier studies indicating that *P. digitatum* is the most economically important postharvest disease of citrus around the world (Eckert and Brown, 1986b). However, on citrus kept in cold storage for long periods, blue mold frequently prevails because it grows better than green mold below 10 °C (Whiteside et al., 1988).

P. agglomerans grew well in wounds on oranges, while it had a limited growth on the surface of the fruit. In fact, the density of culturable *P. agglomerans* agent just after treatment is lower on unwounded fruits than on wounded ones. Bull et al. (1997) found that *P. syringae* strains ESC-10 and ESC-11 survived poorly on the surface of lemons and oranges but were able to effectively colonize wounds and significantly control green and blue molds. Similar results have been found on apples with yeasts as antagonists by Lima et al. (1998) and Usall et al. (2001). These results suggest that the antagonist grows effectively only in wound sites, which are the point of entry of both pathogens. This aspect may be advantageous because, once applied, the biocontrol agent could survive in the microenvironment where it is necessary to prevent disease and decrease to non-detectable or very low concentrations on fruit surface.

The antagonist grew well at 20 °C and 3 °C. This indicates excellent adaptation of the strain CPA-2 to cold storage temperatures, which is an important feature for postharvest biocontrol agents (Wisniewski and Wilson, 1992). The antagonist was tolerant to sodium bicarbonate, but was not affected by salt residues inside rind wounds.

Although these experiments showed that *P. agglomerans* was effective in controlling blue and green mold under laboratory conditions and its efficacy could be improved by a combined treatment with sodium bicarbonate, full-scale commercial evaluation is needed to demonstrate the value of these treatments to the citrus industry. Strain CPA-2 of *P. agglomerans* could be used as effectively on citrus fruits as on pome fruits and the spectrum of activity may be broadened with future research.

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