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Biological control of postharvest pear diseases using a bacterium, *Pantoea agglomerans* CPA-2

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Abstract

Epiphytic microorganisms isolated from the fruits and leaf surfaces of apples and pears were screened for antagonistic activity against *Penicillium expansum* on pears. From 247 microorganisms tested for antagonistic properties against *P. expansum*, a bacterium strain identified as *Pantoea agglomerans* (CPA-2) was selected. This bacterium was very effective against *Botrytis cinerea*, *P. expansum* and *Rhizopus stolonifer*. Complete control at the three tested concentrations (2×10^7, 8×10^7 and 1×10^8 CFU ml^{-1}) was obtained on wounded pears inoculated with 10^3, 10^4 and 10^5 conidia ml^{-1} of *P. expansum* and *R. stolonifer*. At 8×10^7 CFU ml^{-1}, *Pan. agglomerans* reduced *B. cinerea* decay by more than 80% at the three concentrations of the pathogen. In over 3 years of experiments in semicommercial trials, *Pan. agglomerans* provided excellent control against *B. cinerea* and *P. expansum* under cold storage, either in air or in low oxygen atmospheres. Equal control was obtained with *Pan. agglomerans* at 8×10^7 CFU ml^{-1}, as with the fungicide imazalil at commercial doses, against both pathogens. *Pan. agglomerans* grew well inside wounds on pears at both room and cold temperatures and under modified atmospheres. In contrast, it grew poorly on the surface of intact fruit.

Keywords: Biocontrol; *Pantoea agglomerans*; Antagonists; *Penicillium expansum*; *Botrytis cinerea*; *Rhizopus stolonifer*; Pome fruits

1. Introduction

Postharvest fungal decay of pear and apple fruits causes substantial economic losses in Spain (Palazón et al., 1984). Blue mould caused by *Penicillium expansum* is the most important postharvest disease of pome fruits worldwide (Pierson et al., 1971), followed in Spain by *Botrytis cinerea* and *Rhizopus stolonifer* (Palazón et al., 1984).

Postharvest fungicide treatments are the main means of controlling these losses. The development of resistant strains of the pathogens to many fungicides (Spotts and Cervantes, 1986; Viñas et al., 1991, 1993) and the growing concern for human safety and protection of the environment (Norman, 1988; Wisniewski and Wilson, 1992) have resulted in the need to develop other methods to control postharvest decay.

Biological control of storage decay using a microbial antagonist has been considered a desirable alternative to synthetic fungicides. Postharvest biocontrol is especially feasible because harvested fruits are readily accessible to treatment with antagonists and

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many postharvest pathogens of fruits infect through wounds after harvest (Janisiewicz and Jeffers, 1997).

Decay caused by the major postharvest pathogens has been controlled on pome and citrus fruits by bacterial and yeast antagonists, some of which are being commercially developed (Pusey et al., 1988; Janisiewicz and Marchi, 1992; Janisiewicz and Bors, 1995; Chand-Goyal and Spotts, 1997; Viñas et al., 1998).

A screening program was initiated in 1993 to isolate and identify naturally occurring bacteria and yeasts from plant surfaces, which have the potential as biocontrol agents effective against postharvest diseases caused by fungi found in the Mediterranean area, including *P. expansum*, *B. cinerea* and *R. stolonifer*. The bacterium, *Pantoea agglomerans* (CPA-2), was selected. The acute oral toxicity of strain CPA-2 was determined by the Centre d'Investigació i Desenvolupament Aplicat, Barcelona, Spain. At 4.3×10^{11} CFU kg^{-1} live weight of Spargue Dawley rats, no mortality or alterations in growth of the test animals were observed.

The objective of this research was to evaluate the biocontrol potential of strain CPA-2 of *Pan. agglomerans* Gavini et al., 1989 (Viñas et al., 1999), against the three major postharvest pathogens of pears in the Mediterranean area.

2. Materials and methods

2.1. Antagonist isolation

Putative antagonists were isolated from the surfaces of leaves and fruit picked throughout the growing season in 1993. Fruits and leaves were collected and washed in 200 ml sterile 0.05 M phosphate buffer [0.2 M KH_2PO_4, 70 ml (Rectapur, Prolabo, 26 923.298, Fontenay S/B, France); 0.2 M K_2HPO_4, 30 ml (Rectapur, Prolabo, 26 930.293) and deionized water, 300 ml] pH 6.5, on a rotary shaker (Gallenkamp, Lougborough, Leicestershire) for 10 min at 150 rpm. The washings were discarded, and fruit and leaves were washed a second time for 10 min in an ultrasonic bath (Selecta, Abrera, Barcelona, Spain). Washings from sonicated samples (0.1 ml) were plated on nutrient yeast dextrose agar medium [NYDA: nutrient broth, 8 g l^{-1} (Biokar Diagnostics, BK003 Beauvais, France); yeast extract, 5 g l^{-1} (Biokar Diagnostics, 112002); dextrose, 10 g l^{-1} (Rectapur, 24 379.294, Prolabo) and agar, 15 g l^{-1} (Prolabo, 20 768.292)] and incubated for 24–48 h at 25°C. Colonies were isolated on the basis of their different visual characteristics. After isolation, all colonies were purified by single colony isolations after triple restreaking on NYDA medium.

2.2. Production of antagonist and pathogens

For screening of potential antagonists, microorganisms were prepared with three loopfuls of cultures grown for 24–48 h on NYDA and 4.5 ml of 0.05 M phosphate buffer. For further experiments, antagonist suspensions were prepared by growing cultures in nutrient yeast dextrose broth (NYDB) (NYDA without agar) for 24–48 h at 25 ± 1°C with shaking at 150 rpm. The medium was centrifuged (Avanti J-25, Beckman, Palo Alto, CA) at $8315 \times g$ for 10 min and cells were resuspended in deionized water. Desired concentrations were obtained by adjusting the suspension according to a standard curve with a spectrophotometer (CECIL CE 1020, Cambridge) by measuring the optical density at 420 nm.

P. expansum, *B. cinerea* and *R. stolonifer* were isolated from decayed apples after several months in storage and growth on potato dextrose agar medium [PDA: extract of boiled potatoes, 200 ml; dextrose, 20 g (Rectapur, 24 379.294, Prolabo); agar, 20 g (Prolabo, 20 768.292) and deionized water, 800 ml]. To determine the concentration of the conidial suspensions, a haemacytometer (Thoma, Brand, Germany) was used. The conidial suspensions were prepared from 10, 14 and 7-day-old cultures of *P. expansum*, *B. cinerea* and *R. stolonifer*, respectively.

2.3. Fruits

Pears, of the Blanquilla cultivar were obtained from commercial orchards in Lleida, Catalonia, Spain. Fruits were used immediately after harvest or after less than 3 months of storage at 1°C.

2.4. Screening potential antagonists

The method described by Janisiewicz (1987) was used to select microorganisms capable of reducing disease caused by *P. expansum* from a large number

of isolates. The minimum criteria used were reduction in incidence of disease by 50% or more and inhibition of rot diameters by more than 75%. Surface-sterilised pears were wounded, ($3 \times 3 \times 3$ mm^3), at the stem and calyx end. Then, 25 μl of water suspension of an antagonist were pipetted into the wound, followed by inoculation with 20 μl of an aqueous suspension of *P. expansum* conidia (10^4 conidia ml^{-1}). Each fruit constituted a single replicate and each treatment was repeated three times. Lesion diameters were measured after 7 days of incubation at 20 ± 1°C and 85 ± 5% relative humidity (RH) in an air-conditioned room.

2.5. Secondary screenings

The isolate used in the studies was *Pan. agglomerans* strain CPA-2, which provided the most effective results in the preliminary experiments.

To determine the minimum effective concentration of *Pan. agglomerans* against *P. expansum*, *B. cinerea* and *R. stolonifer* on pears, surface-sterilised fruit were wounded as above. Then, 25 μl of aqueous suspensions of strain CPA-2 (2×10^7, 8×10^7 and 1×10^8 CFU ml^{-1}) were applied to each wound. After 1 h, the wounds were inoculated with 20 μl of an aqueous suspension of each pathogen (10^3, 10^4, 10^5 and 10^6 conidia ml^{-1}). Three fruits constituted a single replicate and each treatment was repeated three times. Lesion diameters were measured after 7 days of incubation, at 20 ± 1°C and 85 ± 5% RH in an air-conditioned room. The test was repeated twice.

To determine the effectiveness of the CPA-2 strain against *P. expansum* in pears under cold storage, fruits were treated as above with 2×10^7 and 8×10^7 CFU ml^{-1} of *Pan. agglomerans* and a *P. expansum* concentration of 10^4 conidia ml^{-1}. Ten fruits constituted a single replicate, and each treatment was repeated three times. Fruits were stored in a cold room at 1°C and 90 ± 5% RH for 60 days. The test was repeated twice.

2.6. Semicommercial trials under cold storage conditions

In this study, each fruit was cut in two locations (midway between the calyx and stem end) with a sharp palette knife. The cuts were approximately 1 mm long and 2 mm deep. Fruits were dipped in a *Pan. agglomerans* aqueous suspension at 2×10^7 and 8×10^7 CFU ml^{-1} for 30 s. After 1 h, fruits were dipped again for 30 s in a conidial suspension of *P. expansum* or *B. cinerea* (10^4 conidia ml^{-1}). Twenty fruits constituted a single replicate and each treatment was repeated four times. Lesion diameters were measured after 90 days of cold storage at 1°C and 21% O_2 (air) or 2% O_2–0.7% CO_2 (low oxygen atmosphere). The antagonist efficacy was compared with a control water treatment and the fungicide imazalil (Decozil-S-7.5, Elf Atochem Agri España, Valencia, Spain) at a recommended concentration for standard postharvest treatments (0.5%). This study was conducted during three storage seasons (1996, 1997 and 1998).

2.7. Population dynamics

Population dynamics of CPA-2 were determined on the surfaces of Blanquilla pears, with or without wounds. Two sets of pears were rinsed in fresh water after harvest. One of these sets was wounded as described above (1 mm long and 2 mm deep). All fruit were dipped in a CPA-2 suspension (2×10^7 CFU ml^{-1}) for 30 s. Once dried, fruits were placed on tray packs in plastic boxes and incubated at 20°C and 85 ± 5% RH, or at 1°C and 90 ± 5% RH. Populations of CPA-2 were monitored at 0 (just prior to storage), 24, 48, 72 and 166 h on fruits stored at 20°C, and 0, 15, 30, 45 and 60 days on cold stored fruit.

Twenty pieces of peel surface of 166 cm^2 were removed (including the wounded areas in the case of wounded fruit) with a cork borer and a knife. Pieces of peel were shaken in 200 ml sterile phosphate buffer (pH 6.5) on a rotatory shaker for 20 min at 150 rpm and then sonicated for 10 min in an ultrasonic bath. Serial dilutions of the washings were made and plated on Starch Agar Medium [soluble starch, 3 g l^{-1} (Prolabo 21 152.234); yeast extract, 5 g l^{-1} (Biokar Diagnostics, 112002); peptone, 5 g l^{-1} (Biokar Diagnostics, 117008) and agar, 15 g l^{-1} (Prolabo, 20 768.292)], a specific medium for cultivation of *Pan. agglomerans* (Atlas, 1995) supplemented with imazalil (imazalil sulphate, 99%) (0.5 g l^{-1}) to inhibit fungi. Colonies were counted after incubation at 25°C in the dark for 48 h. Popula-

tion sizes were expressed as CFU cm^{-2} of pear surface. Four fruits constituted a single replicate and each treatment was replicated four times. The experiment was carried out twice.

Population dynamics of *Pan. agglomerans* were also determined on the surface of wounded Blanquilla pears stored at 1°C in an atmosphere of 2% O_2 and 0.7% CO_2. Fruits were wounded and treated as above, except that the *Pan. agglomerans* concentration was 8×10^7 CFU ml^{-1}. *Pan. agglomerans* was recovered, as described above, from pear surfaces after 0, 15, 30, 45 and 60 days in cold storage.

2.8. Statistical treatment

The incidence and severity of decay were analysed by an analysis of variance with SAS Software (SAS Institute, version 6.08, Cary, NC). Statistical significance was judged at the level $P < 0.05$. When the analysis was statistically significant, Duncan's Multiple Range Test was used for separation of means.

Data of antagonist populations (CFU cm^{-2}) were transformed to logarithms to improve the homogeneity of variances.

3. Results

3.1. Isolation of microorganisms and screening potential antagonists

From more than 300 microorganisms isolated, 247 were tested in primary screening against *P. expansum*. More than 60% of these showed some antagonist activity (> 15% reduction in decay diameter) but only 2% of the tested strains reduced the incidence of infected wounds by more than 50%, and reduced lesion diameters by more than 75%. One of these was isolated from the surface of apples and others from the leaves of apple trees.

The most effective microorganism was the strain CPA-2, a bacterium identified as *Pan. agglomerans* by the Centraalbureau voor Schimmelcultures, Delft, Netherlands.

Table 1
Lesion diameters (mm) on pears protected with different concentrations of *Pan. agglomerans* CPA-2 and wounds inoculated with suspensions of *P. expansum*[a]

Concentration of *P. expansum* (conidia ml^{-1})	Concentration of *Pan. agglomerans* (CFU ml^{-1})			
	0	2×10^7	8×10^7	1×10^8
1×10^3	13.8 a	0.0 b	0.0 b	0.0 b
1×10^4	28.7 a	0.0 b	0.0 b	0.0 b
1×10^5	32.5 a	0.0 b	0.0 b	0.0 b
1×10^6	34.4 a	8.2 b	5.3 bc	1.5 c

Different letters in the same row indicate significant differences between means using Duncan's Multiple Range Test ($P < 0.05$).
[a]Means of 18 lesions measured in millimeters after 7 days of incubation at 20°C.

Table 2
Lesion diameters (mm) on pears protected with different concentrations of *Pan. agglomerans* CPA-2 and wounds inoculated with suspensions of *R. stolonifer*[a]

Concentration of *R. stolonifer* (conidia ml^{-1})	Concentration of *Pan. agglomerans* (CFU ml^{-1})			
	0	2×10^7	8×10^7	1×10^8
1×10^3	14.7 a	0.0 b	0.0 b	0.0 b
1×10^4	49.8 a	0.0 b	0.0 b	0.0 b
1×10^5	106.6 a	0.0 b	0.0 b	0.0 b
1×10^6	120.0 a	0.0 b	0.0 b	0.0 b

Different letters in the same row indicate significant differences between means using Duncan's Multiple Range Test ($P < 0.05$).
[a]Means of 18 lesions measured in millimeters after 7 days of incubation at 20°C.

3.2. Secondary screenings

At room temperature, *Pan. agglomerans* strongly inhibited development of *P. expansum* and *R. stolonifer* lesions. No lesions developed on fruit treated with any of the three concentrations of the bacterium and 10^3, 10^4 or 10^5 conidia ml^{-1} of either pathogen (Tables 1 and 2). At a concentration of 8×10^7 CFU ml^{-1}, CPA-2 caused the reduction of *B. cinerea* lesion diameters of 100%, 98% and 81%, with a *B. cinerea* concentration of 10^3, 10^4 and 10^5 conidia ml^{-1}, respectively. Biocontrol activity was not increased by raising the concentration of CPA-2 cells to 1×10^8 CFU ml^{-1} (Table 3).

Under cold storage conditions, the treatment with *Pan. agglomerans* significantly inhibited development ($P < 0.0003$) of *P. expansum* on pears stored at 1°C for 60 days (Fig. 1). At 2×10^7 and 8×10^7 CFU ml^{-1}, the incidence of disease was reduced by

Table 3
Lesion diameters (mm) on pears protected with different concentrations of *Pan. agglomerans* CPA-2 and wounds inoculated with suspensions of *B. cinerea*[a]

Concentration of *B. cinerea* (conidia ml^{-1})	Concentration of *Pan. agglomerans* (CFU ml^{-1})			
	0	2×10^7	8×10^7	1×10^8
1×10^3	39.9 a	2.5 b	0.0 b	2.0 b
1×10^4	60.7 a	15.2 b	1.4 c	3.6 c
1×10^5	71.6 a	42.0 b	13.8 c	16.0 c
1×10^6	80.5 a	58.6 b	49.5 bc	38.6 c

Different letters in the same row indicate significant differences between means using Duncan's Multiple Range Test ($P < 0.05$).
[a]Means of 18 lesions measured in millimeters after 7 days of incubation at 20°C.

91% and 96%, respectively, and severity was reduced by nearly 100% at both concentrations.

3.3. Semicommercial trials under cold storage conditions

The results of semicommercial trials conducted for 3 years with *Pan. agglomerans* against *P. expansum* and *B. cinerea* are shown in Figs. 2 and 3. Significant inhibition was observed in the development of both pathogens. Results were similar in each year.

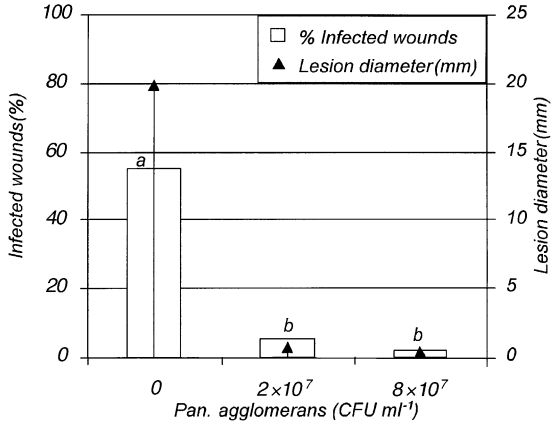

Fig. 1. Suppression of *P. expansum* decay in pears by *Pan. agglomerans* CPA-2. Letters are applied to both percentages of infected wounds and lesion diameters. Columns and lines with the same letter are not significantly different, according to Duncan's Multiple Range Test ($P < 0.05$).

For fruit stored in air, the severity of lesions ($P < 0.001$) and the incidence ($P < 0.0001$) of *P. expansum* were significantly reduced (Fig. 2). Under a low oxygen atmosphere, the best control of *P. expansum* was observed in 1998 (Fig. 2).

No significant differences were observed in the control of *P. expansum* by CPA-2 at 8×10^7

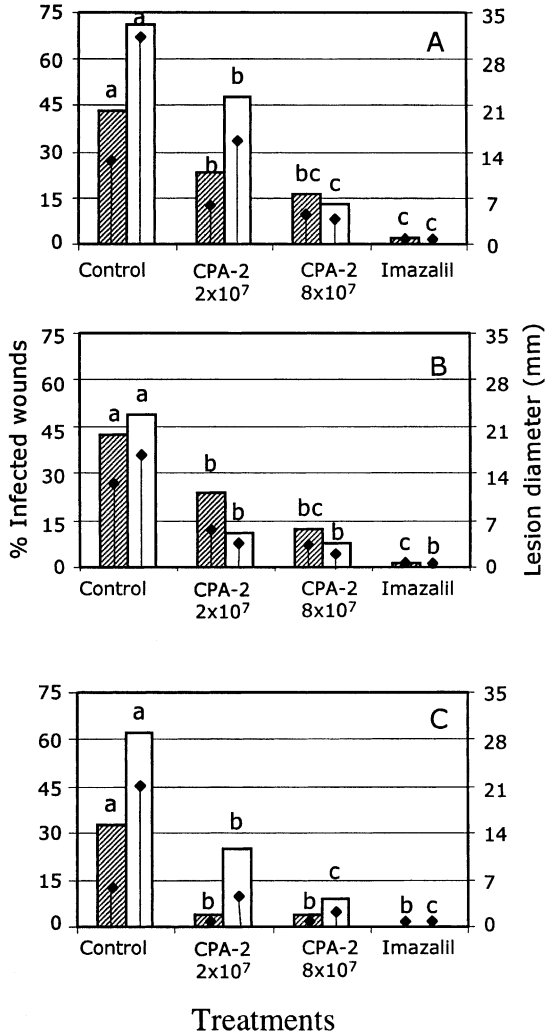

Fig. 2. Suppression of *P. expansum* decay in pears by *Pan. agglomerans* CPA-2 during 1996 (A), 1997 (B) and 1998 (C), and incubation for 90 days at 1°C in air and 21% O_2 (□) or at 2% O_2 and 0.7% CO_2 (▨). Lesion diameters (mm) of *P. expansum* (▲). Within storage conditions, letters are applied to lesion diameters and lines with the same letter are not significantly different, according to Duncan's Multiple Range Test ($P < 0.05$).

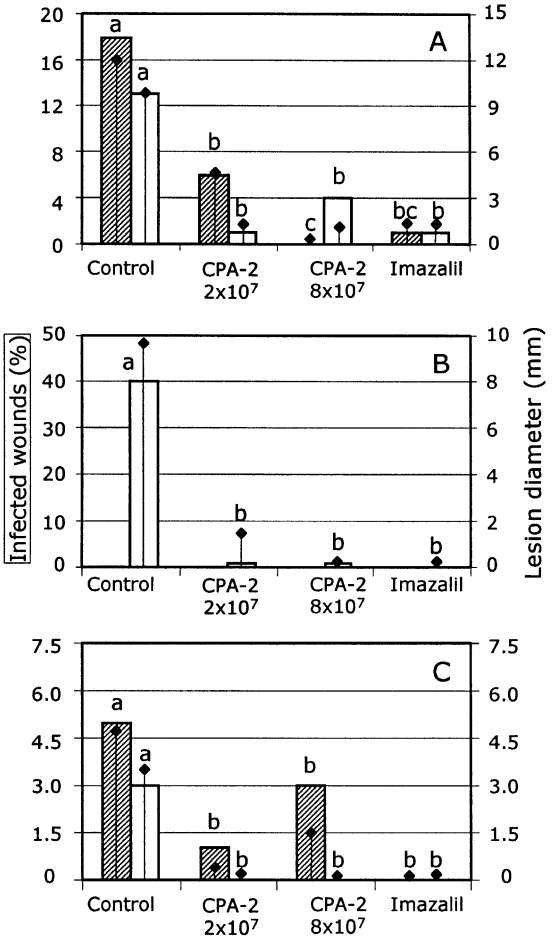

Fig. 3. Suppression of *B. cinerea* decay in pears by *Pan. agglomerans* CPA-2 during 1996 (A), 1997 (B) and 1998 (C). Incubated for 90 days at 1°C in air (□) or at 2% O_2 and 0.7% CO_2 (▨). Lesion diameters (mm) by *B. cinerea* (▲). Within storage conditions, letters are applied to lesion diameters and lines with the same letter are not significantly different, according to Duncan's Multiple Range Test ($P < 0.05$).

CFU ml^{-1} and imidazol under both storage conditions.

Treatment with *Pan. agglomerans* strongly inhibited *B. cinerea* in pears, but the two concentrations of CPA-2 used were not significantly different (Fig. 3). In 1988, control was complete (Fig. 3(C)).

The control of *B. cinerea* under low oxygen atmosphere conditions was less consistent, however, reductions in lesion diameter and wound infection were always highly effective.

No significant differences ($P < 0.0003$) were observed in the control of *B. cinerea* between the treatments with CPA-2 and imidazol.

3.4. Population dynamics

The initial applied concentration of *Pan. agglomerans* was the same in wounded as unwounded fruits (2×10^7 CFU ml^{-1}). However, the population recovered at time 0 in wounded pears was higher (6.6×10^2 CFU cm^{-2}) than in unwounded ones (57 CFU cm^{-2}) (Fig. 4).

In wounded fruit, incubated at 20°C (Fig. 4(A)), the population of CPA-2 reached a maximum after 24 h (5.6×10^4 CFU cm^{-2}) and then decreased, and after 168 h, the population had been reduced to 3.5×10^4 CFU cm^{-2}. In contrast, the population in unwounded pears decreased during the first 48 h, at which time it reached its lowest point (3 CFU cm^{-2}).

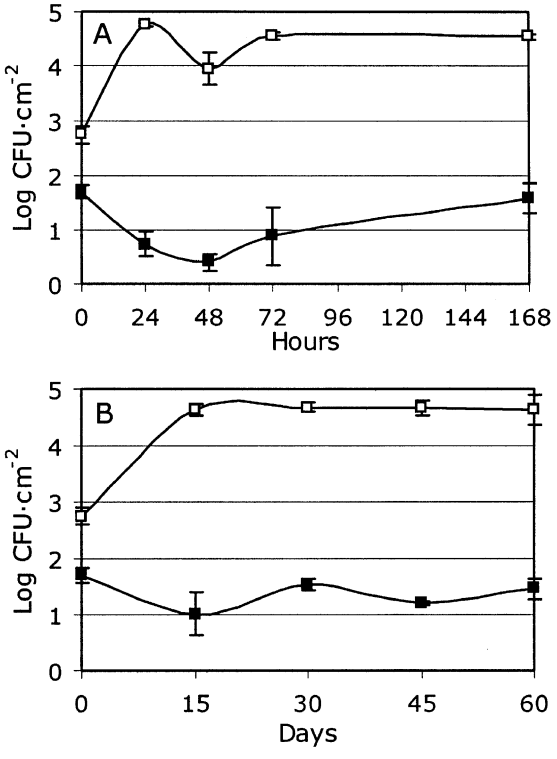

Fig. 4. Population dynamics of *Pan. agglomerans* CPA-2 on surfaces of unwounded (■) and wounded (□) pears incubated at 20°C for 160 h (A), and at 1°C and air for 60 days (B). Points represent the means of four replicates and the vertical bars are standard errors.

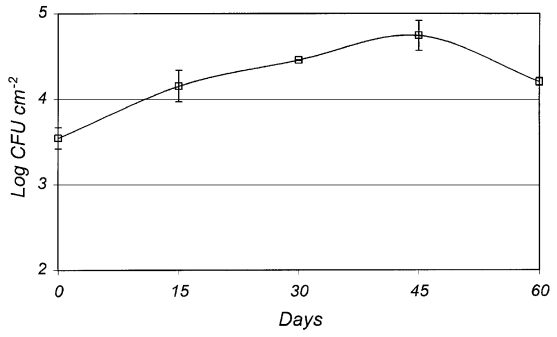

Fig. 5. Population dynamics of *Pan. agglomerans* CPA-2 on the surfaces of wounded pears, incubated at 1°C and low oxygen (2% O_2 and 0.7% CO_2) for 60 days. Points represent the means of four replicates and the vertical bars are standard errors.

At the end of the incubation period, the recovery of cells was similar to that in the initial population.

The population of *Pan. agglomerans* increased 70-fold (4.6×10^4 CFU cm^{-2}) during the first 15 days in wounded fruits incubated at 1°C, and the population remained stable until the end of the study (60 days) (Fig. 4(B)). The population on unwounded fruits was practically stable, declining from 57 CFU cm^{-2} at time 0 to 34 CFU cm^{-2} after 60 days (Fig. 4(B)).

Under cold storage and 2% O_2 plus 0.7% CO_2, the concentration of *Pan. agglomerans* was initially 3.9×10^3 CFU cm^{-2} (Fig. 5). The antagonist population increased gradually and reached a peak of 6.9×10^4 CFU cm^{-2} after 45 days, then decreased slightly, and after 60 days was higher than initial populations.

4. Discussion

From the total of 247 tested microorganisms, only 2% reduced the incidence of infected wounds by more than 50%, and decreased the severity of lesions by more than 75%. This percentage is much lower than the results reported in other studies (Janisiewicz, 1991; Viñas et al., 1998). The most effective isolate, *Pan. agglomerans* strain CPA-2, was selected for further study and showed excellent protection against diseases caused by *B. cinerea*, *P. expansum* and *R. stolonifer* on pears.

Strains of *Pan. agglomerans* have been previously reported as being effective in suppressing diseases of fruit, such as fire blight on apples and pears (Beer et al., 1984a; Johnson et al., 1993; Kearns and Hale, 1995), brown spot on pears (Montesinos et al., 1996), and of *Monilinia oxycocci*, the cranberry cottonball pathogen (Voland et al., 1999). However, *Pan. agglomerans* has not been previously reported as an antagonist of *P. expansum*, *B. cinerea* and *R. stolonifer*.

An important attribute of a successful biocontrol agent is the ability to be efficient at low concentrations (Wisniewski and Wilson, 1992). The CPA-2 strain of *Pan. agglomerans* conformed to this prerequisite by being generally effective against *P. expansum* and *B. cinerea* on pears at a concentration of 2×10^7 CFU ml^{-1}. In 2 out of 3 years, this concentration of *Pan. agglomerans* decreased the severity of *P. expansum* rots by more than 80% and *B. cinerea* rots by more than 87%. This concentration is low enough to be considered suitable for commercial use and is somewhat lower than other recommendations for bacterial biocontrol agents. Janisiewicz (1988) applied *Pseudomonas* sp. at 3×10^8 CFU ml^{-1}, Kampp and Sass (1994) and Mari et al. (1996) recommended the application of 1×10^8 CFU ml^{-1} for *Erwinia* sp. and *Bacillus* sp.

In the present investigation, an increase in *Pan. agglomerans* numbers generally did not provide more effective control of the pathogens in air or in the low oxygen atmosphere at 1°C. In contrast, Pusey and Wilson (1984) and Janisiewicz (1987) reported that increments of antagonist concentration resulted in more effective biocontrol.

Imazalil is one of the most common fungicides used in the postharvest control of fungal rots. The effectiveness of the strain CPA-2 was comparable to that of imazalil at commercial doses, indicating that this biocontrol agent could be used as a substitute for chemicals to control *P. expansum* and *B. cinerea*.

Cold storage in air or under controlled atmospheres is required for the long-term storage of pome fruits, however, atmospheres with less than 2% oxygen may have adverse effects on control microorganisms (Spotts, 1984). However, we found that control of *P. expansum* and *B. cinerea* was equally effective in both storage conditions (21% and 2% oxygen).

Maximum populations of CPA-2 recovered were similar at 1°C (6.2×10^4 CFU cm^{-2}) and at 20°C (5.6×10^4 CFU cm^{-2}). This indicates excellent

adaptation of CPA-2 to cold storage temperatures, a necessary feature for a postharvest biocontrol agent (Wisniewski and Wilson, 1992).

The mechanism by which *Pan. agglomerans* reduces decay is not clear. It has been postulated that *Pan. agglomerans* inhibits plant pathogens by colonisation of the fruit and competition for nutrients (Kempf and Wolf, 1989). This may induce plant defenses or acid conditions (Slade and Tiffin, 1984) or parasitism (Bryk et al., 1998), perhaps by the production of antibiotics (Beer et al., 1984b)

In unpublished observations, we have observed that killed cells of CPA-2 and its culture filtrate were ineffective in reducing the incidence of infection of *P. expansum*, so the presence of living cells is essential. Research is now in progress to test *Pan. agglomerans* in other varieties and fruits, examine formulations and the shelf life of this biocontrol agent, to achieve a commercial product for application in packing houses.

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