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Modes of action of *Pantoea agglomerans* CPA-2, an antagonist of postharvest pathogens on fruits

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Abstract

Pantoea agglomerans CPA-2 is an effective antagonist against the postharvest pathogens *Penicillium digitatum* and *Penicillium italicum* on citrus fruits but its mode of action is unknown. Possible mechanisms studied in this work were antibiosis, induced resistance, competition and production of chitinolytic enzymes. *P. agglomerans* CPA-2 was unable to produce antibiotics or chitinolytic enzymes under the conditions tested. Induction of resistance by *P. agglomerans* CPA-2 was studied in oranges by measuring phenylalanine ammonia lyase and peroxidase enzyme activity in the orange peel at different time points after inoculation with the antagonist and/or the pathogen. No significant augmentation of enzyme activity after inoculation of oranges with *P. agglomerans* CPA-2 in the presence or absence of the pathogen was observed. *P. agglomerans* was effective only when it is in close contact with the pathogens. Competition for nutrients was studied using tissue culture plates with cylinder inserts, which allowed competition for nutrients to be studied without competition for space since physical contact between pathogen and antagonist was avoided. The presence of *P. agglomerans* in the tissue culture wells clearly decreased the germination of *Penicillium* conidia present in the cylinder when diluted orange peel extract or diluted potato dextrose broth was the nutrient source. Germination of *Penicillium* conidia, however, was almost completely inhibited when pathogen and antagonist were in physical contact. These results indicate that competition for nutrients is one of the modes of action of *P. agglomerans* CPA-2, but that physical contact between pathogen and antagonist is important for effective control.

Introduction

Green and blue mould decay caused by *Penicillium digitatum* and *Penicillium italicum*, respectively, accounts for most of the postharvest losses of citrus fruit (Bancroft et al., 1984; Eckert and Brown, 1986). In Europe, these diseases are primarily controlled by the extensive use of fungicides, such as ortho-phenyl phenate, imazalil and thiabendazole. Currently, the use of these chemicals has become restricted because of concerns about the environment and human health as well as the development of resistance to these fungicides among fungal pathogens (Eckert, 1990; Bus et al., 1991; Eckert et al., 1994).

Biological control of fruit decay using microbial antagonists is considered as a desirable alternative to synthetic fungicides. The successful control of major postharvest pathogens through application of biological control agents has been reported (see Janisiewicz and Korsten, 2002). The antagonistic yeast *Candida oleophila* (Aspire) and the bacteria *Pseudomonas syringae* ESC-10 and ESC-11 (Biosave-10 and Bio-save-11) are commercially available for postharvest applications on pome and citrus fruits (Janisiewicz and Jeffers, 1997; Droby et al., 1998).

The modes of action of biological control agents of postharvest diseases are poorly understood. Various

mechanisms have been described, including antibiosis, production of lytic enzymes, parasitism, induced resistance and competition for nutrients and space. Often, more than one mechanism was implicated, but in no case has a sole mechanism been found responsible for biological control (Janisiewicz and Korsten, 2002).

Pantoea agglomerans (Ewing and Fife, 1972) Gavini et al. 1989, comb. nov. (formerly known as *Erwinia herbicola* or *Enterobacter agglomerans*) (Gavini et al., 1989) has been identified as an effective biocontrol agent of postharvest fungal pathogens (Viñas et al., 1999; Ritte et al., 2002); fire blight caused by *Erwinia amylovora* (Ishimaru et al., 1988; Vanneste et al., 1992; Wilson et al., 1992; Wodzinski et al., 1994); damping-off caused by *Pythium* spp. (Nelson, 1988); and several pathogens of cereals such as *Fusarium culmorum*, *Puccinia recondita* f. sp. *tritici* (Kempf and Wolf, 1989) and *P. syringae* pv. *syringae* (Braun-Kiewnick et al., 2000). Recent studies have shown that *P. agglomerans* CPA-2, originally isolated from apple surface, is an effective antagonist of the main fungal pathogens of citrus and pome fruits (Viñas et al., 1999).

Several strains of *P. agglomerans* produce antibiotics *in vitro* (Winkelmann et al., 1980; Ishimaru et al., 1988; Vanneste et al., 1992; Wodzinski and Paulin, 1994; Chernin et al., 1996; Kearns and Hale, 1996; Wright et al., 2001). *P. agglomerans* IC1270 not only produces the antibiotic pyrrolnitrin, but also has chitinolytic activity (Chernin et al., 1995). *P. agglomerans* strain E278Ar can induce resistance to bacterial leaf spot in radish (Han et al., 2000). Competition for nutrients may be important in the control of *Botrytis cinerea* and *Penicillium expansum* by *P. agglomerans* B66 and B90 on apple (Bryk et al., 1998).

The objective of the present study was to determine the mechanisms of biocontrol by *P. agglomerans* CPA-2. The ability of this strain to produce antibiotics and chitinolytic enzymes, to induce resistance in orange fruit and to compete for nutrients with the pathogens *Penicillium digitatum* and *Penicillium italicum* was examined.

Materials and methods

Pathogens

Penicillium digitatum and *Penicillium italicum* were obtained from the UdL-IRTA Centre, Catalonia, Spain and maintained on potato dextrose agar (PDA) medium at 24 °C. A conidial suspension (10^6 ml^{-1}) was prepared by adding 20 ml of sterile water to the surface of 10-day-old cultures, rubbing the surface with a glass rod, and adjusting the concentration with a hemacytometer.

Antagonists

Pantoea agglomerans CPA-2 was obtained from the UdL-IRTA Centre, Catalonia, Spain. The strain was originally isolated from an apple surface (cv. Golden Delicious) (Viñas et al., 1999). *P. agglomerans* A111 was obtained from Prof. Winkelmann, University of Tübingen, Germany. This bacterium produces the antibiotics herbicolin A and B (Greiner and Winkelmann, 1991) and was used as a control in the antibiotic assays. *P. agglomerans* IC1270 was obtained from Prof. Leonid Chernin, the Hebrew University of Jerusalem, Israel. This bacterium produces chitinolytic enzymes and was used as a positive control in assays of chitinolytic activity. Bacterial strains were maintained in 20% glycerol at −80 °C and routinely grown on Luria-Bertani medium (LB; 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl and 15 g of agar per liter) at 28 °C. Bacterial suspensions (10^8 CFU ml^{-1}) were prepared by adding 9 ml of physiological solution (0.85% NaCl) to the surface of a 1-day-old culture, rubbing the surface with a glass rod and adjusting the OD_{595}.

Antibiotic tests

A streak assay was done on PDA, PDA mixed with 10 g l^{-1} orange flavedo tissue powder, and glucose–asparagine medium (GA: 11.5 g K_2HPO_4, 4.5 g KH_2PO_4, 0.12 g $MgSO_4$, 20 g glucose, 0.3 g L-asparagine, 0.05 g nicotinic acid and 15 g agar l^{-1}). An agar disk (diameter 5 mm) from seven days old cultures of the pathogen *Penicillium digitatum* or *Penicillium italicum* was placed at the centre of Petri dishes containing 20 ml agar medium per plate. *P. agglomerans* was streaked at two sides at about 2.25 cm from the centre. The plates were incubated at 25 °C for several days and evaluated for inhibition zones. Ten plates constituted a single replicate and the experiment was replicated twice.

An overlay assay (Wodzinski et al., 1994) was done on PDA, PDA with orange flavedo tissue powder and GA medium. The plates were streak-inoculated in the centre with *P. agglomerans* and incubated at 28 °C for 48 h. The bacteria were scraped off the plates and

the remaining bacteria were killed with chloroform vapours. The plates were then covered with diluted medium (half strength) containing 10^6 conidia ml^{-1} of *Penicillium digitatum* or *Penicillium italicum*. Plates were incubated at 25 °C for seven days and evaluated for inhibition zones. Ten plates constituted a single replicate and each treatment was replicated three times.

Fruits

Valencia oranges were grown in the Baix Ebre and Montsià areas in Tarragona (Catalonia, Spain). After harvest, oranges were stored at 10 °C (for up to five months). Before each experiment, the fruits were surface disinfested with 70% ethanol.

Biocontrol tests

On orange fruits, wounds of about 3 × 3 mm were created with a scalpel and immediately inoculated with 25 µl of *P. agglomerans* CPA-2 (10^8 CFU ml^{-1}). After 2, 24 or 48 h, the same fruits were inoculated with 25 µl of *Penicillium digitatum* (10^6 conidia ml^{-1}) in the same wound (A), in a new wound at a distance of approximately 1 cm from the first wound (B), or in a new wound at the opposite side of the fruit (C). For treatment A, 10 fruits with six wounds per fruit were used, for treatment B, 15 fruits with 4 wounds twice were used and for treatment C, 15 fruits with 2 wounds were used. Fruits were incubated for five days at 20 °C in closed plastic containers before counting the number of wounds showing disease. This experiment was carried out twice. Results were categorized as a dichotomous variable (infected or not infected) and analysed by logistic regression analysis (Agresti, 1990).

Enzyme assays on oranges

Oranges were inoculated with 25 µl of a suspension of *P. agglomerans* (10^8 CFU ml^{-1}), *P. digitatum* (10^5 conidia ml^{-1}) or both (first *P. digitatum* and after 2 h *P. agglomerans*) at 5 wounds on one side of the fruit. The non-inoculated side was used for control samples. At different time points after inoculation (2–72 h) samples were taken from the flavedo tissue around the wounded site, homogenized into acetone, and dried to powder (Ismail and Brown, 1979), which was used for enzyme assays. Three oranges were used for each treatment and the experiment was carried out twice.

Phenylalanine ammonia lyase (PAL) and peroxidase activity were determined by methods modified from Martinez-Téllez and Lafuente (1997). PAL was extracted from 1 g acetone powder with 15 ml 0.1 M sodium borate buffer, pH 8.8, containing 0.02 M β-mercaptoethanol and 50 mg polyvinylpyrrolidone. Proteins were salted out with ammonium sulphate to a final saturation of 46%. The precipitated PAL enzyme was dissolved in 5 ml sodium borate buffer pH 8.8. PAL activity was measured by determining the absorbance at 290 nm of cinnamic acid over a period of 2 h at 40 °C. The reaction mixture contained 100 µl of the purified enzyme extract, 850 µl sodium borate buffer pH 8.8 and 50 µl 0.1 M phenylalanine in sodium borate buffer. PAL activity is expressed as µkats kg^{-1} of protein.

Peroxidase was extracted from 1 g acetone powder with 12 ml 0.1 M Tris–HCl pH 8 containing 0.005 M β-mercaptoethanol. Peroxidase activity was measured at 450 nm during 10 min at 25 °C. The reaction mixture contained 280 µl 0.01 M sodium acetate pH 5.3 containing 0.2% of the substrate guaiacol, 10 µl extract and 10 µl 0.1% hydrogen peroxide. Peroxidase activity was expressed as units per mg protein.

Protein concentrations in the samples were determined with a protein assay kit (Bio-rad) with bovine serum albumin (Sigma) as standard.

In each experiment, data of each time point were statistically analysed using the non-parametric Kruskal–Wallis test followed by the Mann–Whitney test ($P \leq 0.05$).

Competition for nutrients

To test the effect of nutrient depletion by *P. agglomerans* CPA-2 on the germination and growth of *Penicillium digitatum* and *Penicillium italicum* conidia, tissue culture plates with 24 wells per plate and cylinder inserts with a hydrophilic polytetrafluoroethylene membrane (pore size 0.45 µm) attached to the bottom were used (Janisiewicz et al., 2000). Potato dextrose broth (PDB) (20% or 40%) or orange peel extract (1%, 5% or 10%) diluted in physiological solution was dispensed in the wells of culture plates (0.6 ml per well), with or without *P. agglomerans* (10^8 CFU ml^{-1}). *Penicillium digitatum* or *Penicillium italicum* conidia suspension in physiological solution (10^6 conidia ml^{-1}) were dispensed inside the cylinder inserts (0.4 ml per cylinder). The cylinders were placed in the wells and plates were incubated at 24 °C. After 24 or 48 h of incubation, cylinders were removed from

the wells and the membrane was blotted with tissue paper until all the liquid from the inside of the cylinder was absorbed. The membrane was cut out with a scalpel, transferred to a glass slide and spore germination was observed under the microscope. Evaluation was done by estimating the number of germinating conidia per well and dividing the germination into 5 classes: 0: no germination, 1: 0–25% germination, 2: 25–50% germination, 3: 50–75% germination and 4: 75–100% germination.

To determine viability of the conidia after 24 and 48 h of exposure to the antagonist suspension, a parallel set of culture plates with the inserts was prepared as above. After 24 or 48 h of incubation with the antagonist suspension, the inserts containing conidia were removed, the membranes blotted as above, and inserted into new wells containing only the orange peel extract solution or the PDB solution at the corresponding concentrations to those inserted previously. After an additional 24 h of incubation, the inserts were removed, the membranes were blotted, cut and prepared for microscopic observation as described above.

The trial was also carried out without the cylinders, in which the conidia solution was added directly to the well so there was contact between pathogen and antagonist. Evaluation was done by estimating the number of germinating conidia in 100 µl suspension in the presence or absence of the antagonist using the germinating classes described above.

Each experiment was carried out twice with two wells per treatment. Data were statistically analysed using the non-parametric Kruskal–Wallis test followed by the Mann–Whitney test ($P \leq 0.05$).

Production of chitinolytic enzymes

Production of chitinolytic enzymes was tested on the semi-minimal agar medium described by Monreal and Reese (1969) containing 2 g l^{-1} colloidal chitin as the sole carbon source. Plates were inoculated with *P. agglomerans* CPA-2 or *P. agglomerans* IC1270 and incubated for 4 days at 28 °C (Chernin et al., 1995). A clearing zone around the culture was indicative of the production of chitinolytic enzymes.

Results

Antibiotic activity

In the streak assays, a clear inhibition zone was observed for both *Penicillium digitatum* and *Penicillium italicum* on PDA medium or PDA medium with orange flavedo powder inoculated with *P. agglomerans* A111 (data not shown). There was no inhibition zone visible on PDA medium or PDA medium with orange flavedo powder inoculated with *P. agglomerans* CPA-2, irrespective of the pathogen. On GA medium, an inhibition of *Penicillium digitatum* and *Penicillium italicum* was observed only in the presence of *P. agglomerans* A111.

In the overlay assays on PDA medium and GA medium, there was clear inhibition of *Penicillium digitatum* in the presence of *P. agglomerans* A111 but not in the presence of *P. agglomerans* CPA-2. *Penicillium italicum,* however, was not inhibited by *P. agglomerans* A111 or *P. agglomerans* CPA-2 on PDA medium. On GA medium, inhibition of *Penicillium italicum* was observed in the presence of *P. agglomerans* A111 but not in the presence of CPA-2.

Biocontrol tests on oranges

Biocontrol tests with *P. agglomerans* were carried out to see if protection was systemic or local. The results show that there was a protective effect of *P. agglomerans* against *Penicillium digitatum* in orange, but only when there was direct contact between pathogen and antagonist (Figure 1). When *P. agglomerans* and *Penicillium digitatum* were inoculated in the same wound, only 18% of the wounds resulted in disease while inoculation in different wounds led to >90% of wounds resulting in disease.

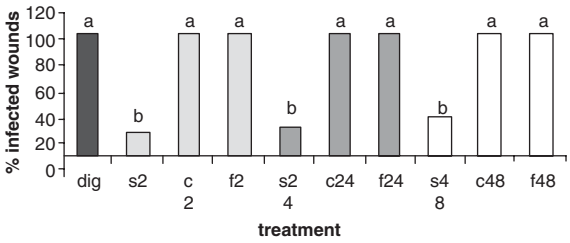

Figure 1. Evaluation of percentage of wounds on oranges showing disease after inoculation with *P. agglomerans* (10^8 CFU ml^{-1}) and *Penicillium digitatum* (10^5 conidia ml^{-1}) after 2 h (light grey bars), 24 h (dark grey bars) or 48 h (hatched bars), in the same wound (s), a new wound at a distance of approximately 1 cm from the first wound (c) or a new wound at the opposite side of the first wound (f). dig: infested control, only inoculated with *Penicillium digitatum* (black bar). Bars with different letters are statistically different by logistic regression analysis ($P \leqslant 0.05$). The experiment was repeated twice with similar results.

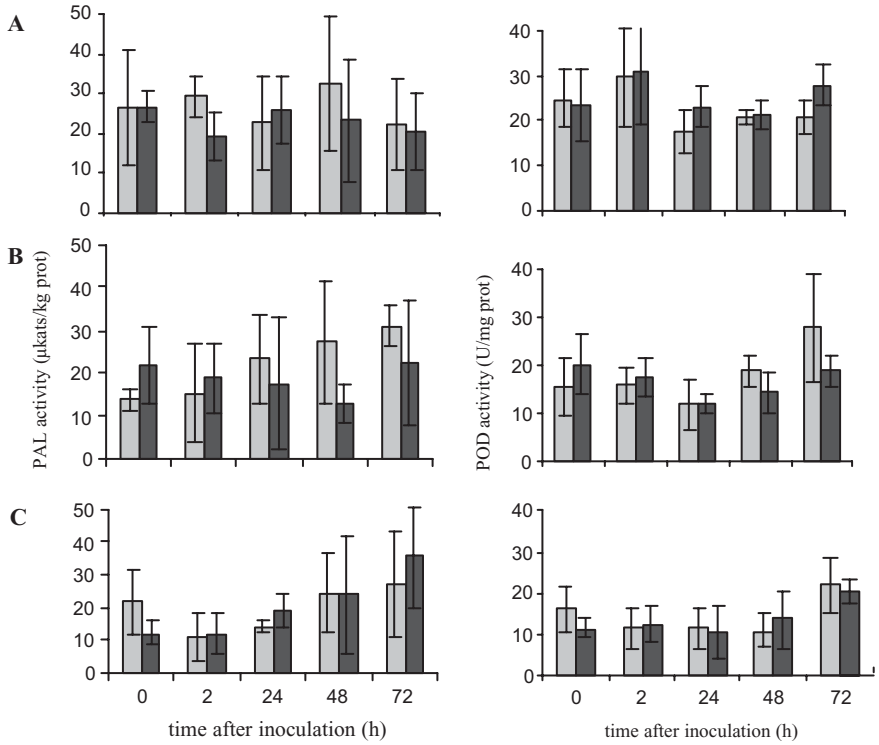

Figure 2. Time course of changes in phenylalanine ammonia lyase (PAL, left) and peroxidase (POD, right) enzyme activity in extracts of orange flavedo wounds (grey bars) treated with *P. agglomerans* CPA-2 10^8 CFU ml^{-1} (A), *Penicillium digitatum* 10^5 conidia ml^{-1} (B) or *P. agglomerans* + *Penicillium digitatum* (C) compared to non-treated control samples (black bars). Extracts were obtained 0, 2, 24, 48 and 72 h after treatment. Each column represents the average activity of three fruits. Bars represent standard deviations. For each time point and pathogen, data were statistically analysed using Kruskal–Wallis followed by Mann–Whitney ($P \leq 0.05$). No significant differences were observed at any time point. Experiments were repeated twice with similar results.

Similar results were found for *P. agglomerans* and *Penicillium italicum* in orange (data not shown). To give *P. agglomerans* more time to induce resistance, the trial was also carried out with inoculation of *Penicillium digitatum* 24 and 48 h after inoculation of *P. agglomerans*. The results were similar; there was only an effect of the antagonist when the pathogen was inoculated in the same wound.

Enzyme activity in orange peel

Previous experiments had shown a great variability in the enzyme activity between oranges (data not shown). Since the effect of *P. agglomerans* in orange was only local in infection trials, we tried to reduce the variability by taking the control samples from the opposite site of the same fruits as the inoculated samples. There was no significant induction of PAL or peroxidase enzyme activity in the orange peel after inoculation with *P. agglomerans* CPA-2 and *Penicillium digitatum*, alone or in combination (Figure 2).

Competition for nutrients with PDB as nutrient source

When *P. agglomerans* was not present in the wells, *Penicillium* conidia germinated at both concentrations of nutrient solution and germination increased with time and nutrient concentration. The presence of the antagonist in the wells prevented germination of the *Penicillium* conidia, regardless of the nutrient concentration (Table 1). Moving the cylinders containing non-germinated conidia from wells with the antagonist to wells without the antagonist resulted in germination of the majority of the conidia (data not shown).

With 20% PDB in the wells, populations of *P. agglomerans* increased from 1×10^8 to 2.14×10^8 with *Penicillium digitatum* in the cylinders and

Table 1. Percent germination of *Penicillium digitatum* and *Penicillium italicum* conidia on membranes cut from cylinders that were inserted for 24 or 48 h into wells containing PDB solutions with (Pan) or without *P. agglomerans* CPA-2

Treatment	24 h						48 h					
	Germination classes[a]					Stat. anal.[b]	Germination classes[a]					Stat. anal.[b]
	0	1	2	3	4		0	1	2	3	4	
P. digitatum												
Control	100	0	0	0	0	a	100	0	0	0	0	a
20% PDB	0	25	75	0	0	b	0	0	0	100	0	b
40% PDB	0	0	25	75	0	c	0	0	0	0	100	c
Control + Pan	100	0	0	0	0	a	100	0	0	0	0	a
20% PDB + Pan	100	0	0	0	0	a	100	0	0	0	0	a
40% PDB + Pan	100	0	0	0	0	a	100	0	0	0	0	a
P. italicum												
Control	100	0	0	0	0	a	100	0	0	0	0	a
20% PDB	0	100	0	0	0	b	0	0	100	0	0	b
40% PDB	0	0	100	0	0	c	0	0	0	0	100	c
Control + Pan	100	0	0	0	0	a	100	0	0	0	0	a
20% PDB + Pan	100	0	0	0	0	a	100	0	0	0	0	a
40% PDB + Pan	100	0	0	0	0	a	100	0	0	0	0	a

[a]Germination classes: 0 = no germination; 1 =< 25% germination; 2 = 25–50% germination; 50–75% germination; 3 = 75–100% germination.

[b]For each time point and pathogen, treatments followed by a different letter are significant different according to Kruskal–Wallis followed by Mann–Whitney ($P \leq 0.05$).

to 2.25×10^8 CFU ml^{-1} with *Penicillium italicum* in the cylinders within 24 h. In 40% PDB, populations increased to 2.63×10^8 and 2.91×10^8 CFU ml^{-1}, respectively. During a second 24 h of incubation, this population remained stable. After blotting the bottom of the membranes and moving the cylinders to other wells containing only nutrient, no measurable growth of the antagonist occurred in the second 24 h of incubation. Thus, no significant carryover of the antagonist occurred from the first to the second incubation well.

When *P. agglomerans* was not present, the *Penicillium* conidia germinated depending on time and nutrient concentration. Presence of the antagonist in the wells prevented germination of the *Penicillium* conidia, irrespective of the nutrient concentration (data not shown).

Competition for nutrients with orange extract as nutrient source

In the absence of *P. agglomerans*, the conidia of *Penicillium digitatum* started to germinate within 24 h in the wells with 1% orange extract (all membranes in germination class 1). At that time, most of the conidia of *Penicillium italicum* had not yet germinated. Germination of the conidia of both pathogens increased as time and nutrient concentration increased. The presence of the antagonist in the wells inhibited germination at 1% orange extract and reduced germination at 5% orange extract during the first 24 h. No inhibition was observed at 10% nutrient concentration (Table 2). After 24 h, populations of *P. agglomerans* in 1% extract decreased slightly from 1×10^8 to 5.85×10^7 CFU ml^{-1} with *Penicillium digitatum* in the cylinders and to 7.76×10^7 CFU ml^{-1} with *Penicillium italicum* in the cylinders. In 5% extract, the *P. agglomerans* increased to 1.32×10^8 and 1.57×10^8 CFU ml^{-1}, respectively. In 10% extract the population increased to 1.46×10^8 and 1.73×10^8 CFU ml^{-1}, respectively. During a second 24 h period of incubation, the population remained stable (data not shown).

Moving the cylinders containing non-germinated conidia from wells with the antagonist to wells without the antagonist resulted in germination of the majority of the conidia (data not shown). After blotting the bottom of the membranes and moving the cylinders

Table 2. Percent germination of *Penicillium digitatum* and *Penicillium italicum* conidia on membranes cut from cylinders that were inserted for 24 or 48 h into wells containing orange peel extract solutions with (Pan) or without *P. agglomerans* CPA-2

Treatment	24 h						48h					
	Germination classes[a]					Stat. anal.[b]	Germination classes[a]					Stat. anal.
	0	1	2	3	4		0	1	2	3	4	
P. digitatum												
Control	100	0	0	0	0	a	100	0	0	0	0	a
1% extract	0	100	0	0	0	b	0	0	0	100	0	b
5% extract	0	0	0	0	100	c	0	0	0	0	100	c
10% extract	0	0	0	0	100	c	0	0	0	0	100	c
Control + Pan	100	0	0	0	0	a	100	0	0	0	0	a
1% extract + Pan	100	0	0	0	0	a	100	0	0	0	0	a
5% extract + Pan	0	0	75	25	0	d	0	0	0	0	100	c
10% extract + Pan	0	0	0	0	100	c	0	0	0	0	100	c
P. italicum												
Control	100	0	0	0	0	a	100	0	0	0	0	a
1% extract	75	25	0	0	0	a	0	0	100	0	0	b
5% extract	0	0	0	0	100	b	0	0	0	0	100	c
10% extract	0	0	0	0	100	b	0	0	0	0	100	c
Control + Pan	100	0	0	0	0	a	100		0	0	0	a
1% extract + Pan	100	0	0	0	0	a	100	0	0	0	0	a
5% extract + Pan	0	0	100	0	0	c	0	0	0	100	0	d
10% extract + Pan	0	0	0	25	75	b	0	0	0	0	100	c

[a]Germination classes: 0 = no germination; 1 =< 25% germination; 2 = 25–50% germination; 50–75% germination; 3 = 75–100% germination.

[b]For each time point and pathogen, treatments followed by a different letter are significant different according to Kruskal–Wallis followed by Mann–Whitney ($P \leqslant 0.05$).

to other wells containing only nutrient, no measurable growth of the antagonist occurred in the second 24 h of incubation. Thus, no significant carryover of the antagonist occurred from the first to the second incubation well.

When the conidial suspension was added directly into the well with *P. agglomerans*, germination of *Penicillium* conidia was inhibited in 1% and 5% orange extract during the first 24 h and was greatly reduced in 10% orange extract (conidia in all the wells were in germination class 1). After 48 h, there was also little germination in 5% extract (conidia in all the wells were in germination class 1) but germination at 10% had only slightly increased (conidia in 75% of the wells were in germination class 1; conidia in 25% of the wells were in germination class 2) (Table 3).

Production of chitinolytic enzymes

After four days of incubation, *P. agglomerans* IC1270 produced clearing zones, while no clearing zones were observed for *P. agglomerans* CPA-2 on semi-minimal agar medium supplemented with colloidal chitin.

Discussion

Pantoea agglomerans CPA-2 is an effective antagonist against postharvest pathogens on citrus and pome fruits (Viñas et al., 1999; Nunes et al., 2001, 2002). However, the mechanism(s) by which *P. agglomerans* reduces decay is (are) not clear. In this study, four possible mechanisms were studied: antibiosis, induced resistance, competition for nutrients and production of chitinolytic enzymes. The data suggest that competition for nutrients plays a role in the biocontrol of *P. agglomerans* CPA-2 on citrus fruits. Neither induced resistance nor production of chitinolytic enzymes seem to be involved.

Several strains of *P. agglomerans* produce antibiotics or bacteriocins, which inhibited *Erwinia amylovora in vitro*. Strain C9-1 produces herbicolin

Table 3. Percent germination of *Penicillium digitatum* and *Penicillium italicum* conidia after 24 or 48 h incubation in orange peel extract solutions with (Pan) or without *P. agglomerans* CPA-2

Treatment	24 h						48h					
	Germination classes[a]					Stat. anal.[b]	Germination classes[a]					Stat. anal.[b]
	0	1	2	3	4		0	1	2	3	4	
P. digitatum												
Control	100	0	0	0	0	a	100	0	0	0	0	a
1% extract	0	100	0	0	0	b	0	0	0	100	0	b
5% extract	0	0	0	0	100	c	0	0	0	0	100	c
10% extract	0	0	0	0	100	c	0	0	0	0	100	c
Control + Pan	100	0	0	0	0	a	100	0	0	0	0	a
1% extract + Pan	100	0	0	0	0	a	100	0	0	0	0	a
5% extract + Pan	100	0	0	0	0	a	0	100	0	0	0	d
10% extract + Pan	0	100	0	0	0	b	0	75	25	0	0	d
P. italicum												
Control	100	0	0	0	0	a	100	0	0	0	0	a
1% extract	100	0	0	0	0	a	0	75	25	0	0	b
5% extract	0	0	0	0	100	b	0	0	0	0	100	c
10% extract	0	0	0	0	100	b	0	0	0	0	100	c
Control + Pan	100	0	0	0	0	a	100	0	0	0	0	a
1% extract + Pan	100	0	0	0	0	a	100	0	0	0	0	a
5% extract + Pan	100	0	0	0	0	a	0	100	0	0	0	b
10% extract + Pan	0	100	0	0	0	c	0	100	0	0	0	b

[a]Germination classes: 0 = no germination; 1 =< 25% germination; 2 = 25–50% germination; 50–75% germination; 3 = 75–100% germination.

[b]For each time point and pathogen, treatments followed by a different letter are significant different according to Kruskal–Wallis followed by Mann–Whitney ($P \leqslant 0.05$).

O and I (Ishimaru et al., 1988) while strain Eh 318 produces pantocin A and B antibiotics (Wodzinski et al., 1990, 1994; Wright et al., 2001). The strains Eh1087, Eh252 and P10c produce antibiotics that have not been identified (Kearns and Hale, 1996; Kearns and Mahanty, 1998; Vanneste et al., 1992; 2002). Recently, it was shown that antibiosis is an important mechanism of biocontrol of fire blight by *P. agglomerans* Eh252 (Stockwell et al., 2002). El-Goorani et al. (1992) tested nine antibiotic-producing strains of *P. agglomerans*. These strains strongly inhibited the growth of various *Erwinia* species, but they did not inhibit the growth of *Penicillium digitatum* or other tested fungi. Another antibiotic, pyrrolnitrin, produced by *P. agglomerans* IC1270 did have a fungicidal effect against fungi such as *B. cinerea* (Chernin et al., 1996), *Penicillium expansum*, *Monilinia fructicola* and *Rhizopus stolonifer* (Ritte et al., 2002). Pyrrolnitrin is also produced by *Burkholderia cepacia* LT412W (=*P. cepacia*), an effective antagonist of postharvest fungal pathogens on pome (Janisiewicz et al., 1991) and citrus fruits (Smilanick and Denis-Arrue, 1992). These pathogens were also controlled by application of purified pyrrolnitrin (Janisiewicz et al., 1991). *B. cinerea* was also inhibited by the herbicolin A produced by *P. agglomerans* A111 (Winkelmann et al., 1980). Strain Eh B247 also produces herbicolin A and inhibits the growth of *F. culmorum* on wheat (Kempf and Wolf, 1989; Kempf et al., 1993).

In this study, no inhibition of the pathogens' growth was observed *in vitro* in the presence of *P. agglomerans* CPA-2, but inhibition was observed for *Penicillium digitatum* and to a lesser extent for *Penicillium italicum* in the presence of *P. agglomerans* A111. Similar inhibition zones for *Penicillium digitatum* were observed on PDA medium in the presence of the pyrrolnitrin-producing *P. agglomerans* IC1270 (unpublished results). These results show that our *in vitro* biotest is relevant to assess the activity of antibiotics such as herbicolin A and pyrrolnitrin. The lack of antagonism observed for *P. agglomerans* CPA-2 suggests that in our experimental conditions strain CPA-2 does not

exert any antibiotic activity. Since the antagonistic activity against *Penicillium digitatum* and *Penicillium italicum* did not seem related to antibiosis, the possibility that CPA-2 induces resistance in orange fruits was investigated.

A third possible mechanism is competition for nutrients, which was tested by a non-destructive *in vitro* method (Janisiewicz et al., 2000) which allows the study of competition for nutrients without any competition for space (Janisiewicz et al., 2000). The observed inhibition of conidia germination on the membranes appears to be due to lack of nutrients, since conidia on the membranes readily germinated when they were moved to fresh nutrient solutions without antagonist. Competition for nutrients also seems to be implicated in the control of *Penicillium* spp. by the bacterial antagonists *P. syringae* strains ESC-10 and ESC-11 (Bull et al., 1997) and *Burkholderia cepacia* LT412W (Smilanick and Denis-Arrue, 1992) and antagonistic yeasts such as *Debaryomyces hansenii* (Droby et al.,1989); *Pichia guilliermondi* (Arras et al., 1998) and *Aureobasidium pullulans* (Janisiewicz et al., 2000; Castoria et al., 2001).

When orange peel extract was used as nutrient source, a difference in germination rate was observed between contact and no contact trials. When there was no contact, the antagonist prevented germination at low nutrient concentrations but not at higher nutrient concentrations. This indicates that competition for nutrients may play a role at low but not at high nutrient concentrations. When there was direct contact between pathogen and antagonist, however, germination in orange extract was lower than when there was no contact. This indicates that besides competition, another mechanism could be active. Bryk et al. (1998) observed a similar phenomenon for *P. agglomerans* strains B66 and B90, which controlled blue and grey mould of apple. A high inhibition of spore germination of *Penicillium expansum* in the presence of the bacteria was evident in diluted but not in undiluted apple juice, indicating that the availability of nutrients played a critical role in the inhibition. In addition *P. agglomerans* strains B66 and B90 demonstrated taxis to the conidia and germ tubes of *B. cinerea* and *Penicillium expansum* and caused lysis of the germ tubes in diluted apple juice (Bryk et al., 1998). A similar mechanism could account for the lower germination of *Penicillium* conidia when there is contact with *P. agglomerans* CPA-2 but this remains to be investigated.

In conclusion, *P. agglomerans* CPA-2 is an effective antagonist of *Penicillium digitatum* and *Penicillium italicum* in oranges but only when antagonist and pathogen are in close contact. Competition for nutrients appears to be an important mechanism in the antagonistic activity of *P. agglomerans* CPA-2 but no evidence was found for a possible role of antibiosis or induced systemic resistance.

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