Control of Green Mold of Lemons with *Pseudomonas* Species

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ABSTRACT

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Applications of *Pseudomonas cepacia* reduced postharvest green mold (*Penicillium digitatum*) by more than 80% in lemon fruit (*Citrus limon*) compared with controls. The bacterium grew rapidly in wounds and caused no visible injury to the fruit. Decay was controlled if *P. cepacia* was applied within 12 hr or less after inoculation. The fungicides imazalil and thiabendazole were better eradicants; they were effective when applied 24 hr after inoculation. Other pseudomonads were not as effective as *P. cepacia*. Inhibition of fungal growth in vitro by *P. cepacia* reportedly is caused by the antibiotic pyrrolnitrin. Spores of *P. digitatum* did not germinate in potato-dextrose broth amended with 0.1 μg/ml pyrrolnitrin. However, decay caused by pyrrolnitrin-resistant mutants of *P. digitatum*, which could germinate in pyrrolnitrin at 60 μg/ml, was controlled by *P. cepacia*. Washed, viable cells of *P. cepacia* and *P. corrugata* reduced decay whereas cell-free culture fluids did not. *P. fluorescens* did not inhibit *P. digitatum* growth in vitro yet it reduced decay by about 70%. Antibiotics may not comprise the entire mode of action of pseudomonads on citrus.

Penicillium digitatum (Pers.:Fr.) Sacc. causes green mold of citrus, an important postharvest storage problem wherever citrus are grown, with annual losses up to $50 million in California alone (16). This disease is controlled primarily by applications of ortho-phenyl phenate, imazalil, and thiabendazole, although pathogen resistance to these chemicals has progressively diminished their efficacy (14). Public concerns about health risks associated with the ingestion of pesticide residues also threaten the availability of fungicides in the future. Long-term dietary risks posed by synthetic pesticides are uncertain (1) and generally poorly supported when quantified (3), but public perceptions of hazard are real and threaten continued pesticide use.

Alternatives to fungicides for control of green mold of citrus include physical treatments (24,43), careful handling to minimize creation of wound infection courts required for successful infection (11,41), packinghouse sanitation to reduce inoculum density (5), degreening at 30 C under moist conditions to enhance resistance to infection (8), and biological control. Many organisms can protect wounds from subsequent challenge inoculations including *Pseudomonas* spp. (33,47), *Myrothecium roridum* Tode:Fr. and *M. verrucaria* (Albertini & Schwein.) Ditmar:Fr. (2), *Trichoderma viride* Pers.:Fr. (7,12), *Rhodotorula* sp. (12), *Aureobasidium pullulans* (de Bary) G. Arnaud (47), *Bacillus* spp. (33,39), and *Debaryomyces hansenii* (Zopf) Lodder & Kreger van Rij (10,13,47), subsequently identified as *Candida guilliermondii* (Castellani) Langeron & Guerra (34). Most of these organisms reduce decay initiated at wounds if applied before or with pathogen spores. The *Myrothecium* spp. were an exception; they both protected wounds and controlled decay up to 18 hr after inoculation (2).

In commercial citrus culture, wounds are inflicted and become inoculated during harvest and subsequent handling. Therefore, an application to fruit on packing lines of a decay control agent that both eradicates infections and protects wounds is of value. Our objectives were to determine the after-inoculation efficacy of selected *Pseudomonas* spp. in controlling green mold of citrus in wound-inoculated lemon fruit and to investigate the role of antifungal antibiotic production in decay control.

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MATERIALS AND METHODS

Fruit. Lemons (*Citrus limon* (L.) N. L. Burm.) used in all experiments were California- or Arizona-grown (cv. Eureka) lemons hand-selected from field bins after harvest, before any commercial postharvest treatments were applied. Lemons selected were light green to pale yellow in color and were stored no longer than 2 wk after harvest at 10 C before use.

Inoculum. Potato-dextrose agar (PDA) petri dishes were inoculated with *P. digitatum* isolate M6R (from J. W. Eckert, University of California, Riverside) and incubated at 20 C for 1-2 wk. Spores were rubbed from the agar surface with a glass rod after a small volume of sterile 0.05% Triton X-100 was added. The spore suspension was passed through two layers of cheesecloth and diluted with sterile water to an optical density of 0.1 *A* at 420 nm, approximately 10^6 spores per milliliter. This is a recommended concentration for the evaluation of postharvest treatments to control green mold (15). For inoculation, fruit were wounded with a saw-inoculation device (35), in which a saw blade spun continuously through the inoculum solution and made an injury (2 mm deep × 10 mm long) on each fruit, or with a stainless steel tool with a tip (2.5 mm long × 1.0 mm wide) that had been immersed briefly in the spore suspension. The shallow wounds made by these devices penetrated the albedo tissue, but not the juice sacs, and simulated natural inoculation (15). One wound was made per fruit. Data were recorded as the percentage of decayed fruit. Sporulation was evaluated on a scale of 0-5 where 0 = negligible sporulation and 5 = dense sporulation over the entire fruit surface (15). When applied, statistical analysis employed arcsine-transformed percentages for analysis of variance followed mean separation by Duncan's new multiple range test.

Antagonistic treatments. *Pseudomonas cepacia* (ex Burkholder) Pallerni and Holmes isolate LT412W from apple leaves, a gift of W. J. Janisiewicz (Kearneysville, WV); *P. corrugata* Roberts and Scarlett, isolated from tomato pith (ATCC 29736); *P. aeruginosa* (Schroeter) Migula, a gift of E. E. Harding (California State University, Fresno); and *P. fluorescens* (Trevisan) Migula (ATCC 13525) were tested for green mold of citrus control. The identities of the cultures were confirmed by fatty acid analysis. The isolates had similarity indices exceeding 0.79, an excellent match, to the same genus and species of each among a library of more than 6,000 aerobic bacteria including 50 *Pseudomonas* spp. (Microcheck, Inc. Northfield, VT). All isolates were stored at −80 C in 73 μl of dimethyl sulfoxide and 937 μl of dense bacterial culture in nutrient broth until needed. The bacteria were cultured for about 72 hr at 23-26 C in shake culture in Erlenmeyer flasks half-filled with nutrient broth (5 g of pancreatic digest of gelatin and 3 g of beef extract per liter). At this time, the pH of the cultures was 7.6-7.9 and they

contained 10^8 to 10^9 colony-forming units (cfu) per milliliter. Populations were enumerated by dilution in 1% bactopeptone and plating on nutrient agar. Population densities were routinely calculated from absorbance at 630 nm. Wounds were treated by immersion of the entire fruit for 1 min in the antagonist culture or by direct application of 25 μl of the antagonist culture to wounds.

Fruit treatments. Lemon fruit at 20 C were saw-blade inoculated 2 hr before immersion in 3-day-old nutrient broth cultures of *P. cepacia, P. corrugata, P. aeruginosa,* or *P. fluorescens.* Each culture contained between 10^8 and 10^9 cfu/ml and was in late log-phase growth. A randomized complete block design with four replicates of 100 fruit per replicate was used. After 1, 2, and 3 wk, the decayed fruit were counted. The experiment was done once.

The minimum colony-forming units of the biocontrol bacteria necessary to control decay was determined by direct application of a nutrient broth dilution series of 3-day-old bacterial cultures to wounds 2.5 mm deep × 1 mm wide in lemon fruit 2 hr after inoculation with 10 μl of 10^6 spores per milliliter of *P. digitatum.* A single sample of 40 fruit was used for each dilution. After 1 wk, the decayed fruit were counted. The experiment was repeated twice.

The influence of fruit color on decay control was assessed with saw-blade inoculation of fruit of three color classes (green, light green, and yellow) 2 hr before they were immersed in 3-day-old cultures of *P. cepacia, P. corrugata, P. aeruginosa,* or *P. fluorescens.* The lemons had been hand-selected from within field bins containing fruit of mixed color classes. A randomized complete block design with four replicates of 25 fruit per replicate was used. After 1, 2, and 3 wk, the decayed fruit were counted. The experiment was done once.

The growth of *P. cepacia* in wounds or on the surface of lemon fruit was investigated by the application of 1.5 × 10^6 cfu in 25 μl of water to a puncture wound 2.5 mm deep by 1.0 mm wide or on the unwounded fruit surface. Sterile water was applied as a control. Two sites each on three fruit where the bacterium or water had been applied were aseptically excised with a scalpel after 0.25, 24, 48, 72, and 96 hr, suspended in 10 ml of nutrient broth and homogenized, and the number of colony-forming units was determined by dilution plating on nutrient agar. The experiment was repeated twice.

The ability of *P. cepacia* and *P. corrugata* applications to control green mold after inoculation was determined with saw-blade inoculation of lemons that were immersed 2, 6, 12, and 24 hr later in 3-day-old cultures of the antagonists. A randomized block design was used. Controls were inoculated fruit immersed in water for each treatment period. After 1 wk, the decayed fruit were counted. The experiment was repeated twice with *P. cepacia* with two replicates of 40 fruit each, whereas *P. corrugata* was tested once with four replicates of 25 fruit each.

The effect of application of *P. cepacia* and *P. corrugata* for control of green mold was compared with that provided by imazalil and thiabendazole. Saw-blade inoculated fruit were treated by 1-min immersion in 3-day-old cultures of the bacteria or 1,000 μg/ml of the fungicides 24 hr after inoculation. A randomized complete block design was used with four replicates of 20 fruit per replicate. The decayed fruit were counted after 3 wk. The experiment was done once.

Antifungal antibiotic production by *Pseudomonas* spp. The direct effect of the four pseudomonads on growth of *P. digitatum* in vitro was examined. Spores of *P. digitatum* were rubbed from the agar surface of cultures grown for 1 wk on PDA at 20 C. The spores were mixed with PDA at 40 C and poured into 10-cm-diameter petri dishes. A 10-μl aliquot containing 10^8 cfu/ml of washed cells of *P. cepacia, P. corrugata, P. aeruginosa,* or *P. fluorescens* was placed on the surface of the solidified agar. The cells were washed by centrifugation (14,000 *g* for 30 sec) and resuspension in fresh nutrient broth. This was repeated five times. The presence and size of clear zones around the bacterial colonies were noted after 48 hr at 20 C. The experiment was repeated twice.

Lemons were treated with cultures, washed cells, and cell-free culture fluids of *P. cepacia, P. corrugata, P. aeruginosa,* or *P. fluorescens* to determine if decay control by the antagonists was the result of antifungal metabolites produced in the nutrient broth cultures before the bacteria were applied to fruit, or if decay control resulted from some other factor, such as de novo antifungal compound synthesis or nutrient competition. Lemons were puncture-inoculated with 10^6 spores per milliliter of *P. digitatum* as previously described. One of the following was applied in a 25-μl volume to the inoculated wound: 1) culture of the pseudomonads at a concentration of 10^9 cfu/ml; 2) pseudomonad cells that had been centrifuged and resuspended in fresh nutrient broth five times at a concentration of 10^9 cfu/ml; 3) culture fluid collected from centrifugation and sterilized with a 0.2-μm pore size filter; and 4) sterile water. A randomized complete block design with four replicates of 20 fruit per replicate was used. After 1 wk, the decayed fruit were counted. The experiment was repeated twice.

The possible role of the antibiotic pyrrolnitrin in the control of *P. digitatum* by *P. cepacia* was investigated by the use of pyrrolnitrin-resistant isolates of the pathogen. To obtain pyrrolnitrin-resistant isolates of *P. digitatum,* we dissolved crystalline pyrrolnitrin (a gift of J. Roitman, Albany, CA) in ethanol and dispensed the solution in PDA at 40 C to a final concentration of 1 μg/ml. Spores were rubbed from the surface of 1-wk-old PDA cultures of *P. digitatum* M6R, 10^6 spores were applied to each plate, and the plates were incubated for 1 wk at 20 C. Single-spore isolations were made from colonies that developed; approximately one colony per plate was observed. The presumptive resistant isolates were evaluated in microplates containing 2 ml of potato-dextrose broth amended with pyrrolnitrin at 0, 0.1, 0.5, 2.5, 5, 10, 20, 40, and 60 μg/ml. The medium contained 0.05% (w/v) Triton X-100 and 0.01% (w/v) dimethyl sulfoxide to increase the solubility of pyrrolnitrin. After 24 hr of incubation at 20 C, the germination and germ tube size of spores of the isolates were assessed by light microscopy. Lemons were saw-blade inoculated with 10^6 spores per milliliter of pyrrolnitrin-sensitive *P. digitatum* isolate M6R and three isolates selected for resistance to pyrrolnitrin. Two or 6 hr later, the lemons were immersed in 3-day-old cultures of *P. cepacia* for 1 min. A randomized complete block design with 20 replicates of five fruit per replicate was used. After 1, 2, and 3 wk, the decayed fruit were counted. The experiment was repeated twice, first with a 6-hr delay between inoculation and treatment, and second with a 2-hr delay between inoculation and treatment, each time with a different pyrrolnitrin-resistant isolate of *P. digitatum.*

RESULTS

Decay after 3 wk following the immersion of uninoculated lemons in water or inoculated lemons in water, *P. corrugata, P. aeruginosa, P. fluorescens,* or *P. cepacia* was 12.9, 94.2, 73.3, 57.8, 21.7, and 8.1%, respectively. *P. cepacia* controlled decay most effectively, but all the *Pseudomonas* spp. reduced decay significantly compared with the inoculated control. The number of decayed fruit did not change among all the treatments after 1, 2, or 3 wk of incubation except for those treated with *P. corrugata,* where decay was 45.3, 71.4, and 73.3% at each inspection, respectively. *P. corrugata* primarily delayed the onset of symptoms rather than controlled decay. A concentration of greater than 10^7 cfu/ml of each bacterium was necessary to reduce decay to less than 60% compared with controls on puncture-inoculated fruit (Fig. 1).

Decay control by the pseudomonads was slightly but significantly ($P = 0.01$) superior on the green or light green lemons compared with the yellow lemons (Fig. 2). *P. cepacia* and *P. fluorescens* reduced decay equally on light green and green lemons; however, *P. fluorescens*

was significantly ($P = 0.01$) inferior on yellow lemons.

P. cepacia grew rapidly in wounds, increasing about 100-fold to 10^7 cfu per wound in 48 hr (Fig. 3). Wounds to which water was applied contained about 100 cfu per wound or less of unidentified yeasts and bacteria. When applied to the unwounded lemon surface, *P. cepacia* could not be recovered after 24 hr at 20 C.

P. cepacia and *P. corrugata* significantly ($P = 0.01$) controlled decay when applied up to 12 hr after inoculation of lemons with spores of *P. digitatum* (Fig. 4). Decay control by *P. cepacia* was superior to *P. corrugata* at every inoculation period tested.

Decay of untreated lemons or after the immersion of inoculated lemons in water, nutrient broth, *P. corrugata, P. cepacia,* thiabendazole, or imazalil was 1.1, 62.3, 67.3, 58.6, 46.7, 6.7, and 0%, respectively. Imazalil and thiabendazole controlled 24-hr-old green mold infections and did not differ significantly. Of the bacteria, only *P. cepacia* reduced decay slightly but significantly, whereas *P. corrugata* did not control decay.

After 48 hr of incubation at 20 C on PDA with *P. digitatum*, inhibition zones 10, 7, and 1 mm in width were observed around colonies of *P. corrugata, P. cepacia,* and *P. aeruginosa*, respectively. No zone of inhibition of fungal growth was present around colonies of *P. fluorescens.*

The decay suppression by *P. cepacia* and *P. corrugata* was correlated with the bacterial cells because their filter-sterilized, cell-free culture fluids did not control decay (Table 1). Both the cell-free culture fluids and the washed cells of *P. aeruginosa* and the washed cells of *P. fluorescens* reduced postharvest decay significantly, although not as well as unwashed bacterial cells in 3-day-old culture fluids.

Three pyrrolnitrin-resistant isolates derived from *P. digitatum* M6R, designated R1, R2, and R3, germinated more than 90% in pyrrolnitrin at 60 μg/ml, whereas spores of the sensitive isolate M6R did not germinate in pyrrolnitrin at 0.1 μg/ml. Germ tube size of the resistant isolates was not influenced by pyrrolnitrin and was not different from that of sensitive isolate M6R in potato-dextrose broth alone.

Pyrrolnitrin-resistant and -sensitive isolates of *P. digitatum* were equally controlled by *P. cepacia* (Fig. 5). Isolate R1 was less virulent and sporulation on lemons was delayed about 2 days in comparison with the parental isolate M6R of *P. digitatum*. The percent decay and the sporulation of lesions after inoculation of lemons with isolates R2 and R3 was not different from that of isolate M6R. Decay control was significantly ($P = 0.05$) better in the second experiment when the bacterium was applied 2 hr after inoculation compared with 6 hr after inoculation in the first experiment. *P. cepacia* reduced decay by 94.1 and 58.4% compared with the controls when applied 2 or 6 hr after inoculation, respectively. The relatively poor control of the 6-hr-old inoculations was not observed in other tests.

DISCUSSION

Pseudomonads could reduce postharvest decay substantially, even if applied 12 hr after inoculation, although they were inferior in efficacy to the fungicides imazalil and thiabendazole, which are used commercially on citrus. The most efficacious biological control agent identified in the present work, *P. cepacia*, controlled postharvest citrus green mold in an earlier study (47). *P. cepacia* is a gram-negative, nonfluorescent pseudomonad of extreme genetic diversity (38,45). It is a ubiquitous, primarily soil-inhabiting organism with little tolerance to desiccation that can only survive in low populations on leaf surfaces (4,29,30,32). Knudsen and Hudler (28) reported the biocontrol activity of pseudomonads requires higher populations than are self-sustaining on plant surfaces. In the present study, *P. cepacia* survived and increased rapidly in wounds on lemon fruit, although the colony-forming units required for decay control were high. Without wounds to colonize, it survived poorly on the surface of the lemons. *P. cepacia* also controls postharvest decay initiating from wounds of fruits of apple (26), blueberry (44), cranberry (44), peach (40), and pear (26).

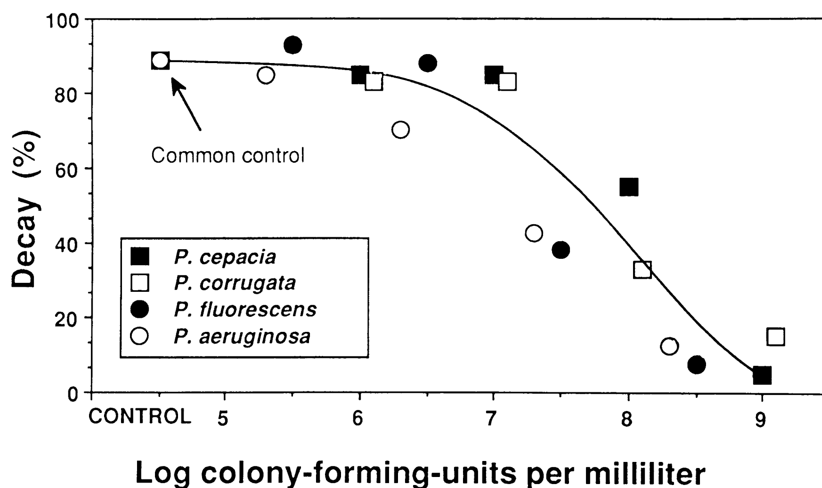

Fig. 1. Influence on postharvest decay of lemons of the colony-forming units per milliliter of *Pseudomonas* spp. applied to lemon wounds puncture-inoculated 2 hr before with *Penicillium digitatum*. A common control was shared among all treatments. Decay was assessed after 1 wk at 20 C.

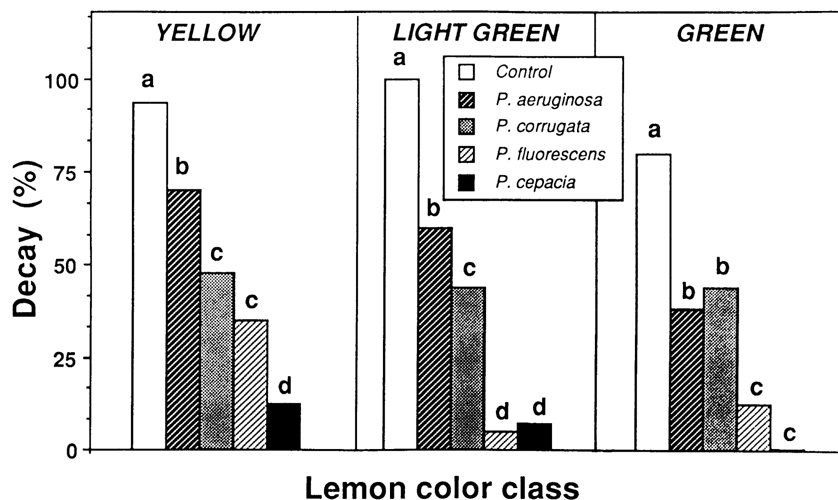

Fig. 2. Postharvest decay of lemons after immersion in *Pseudomonas* spp. cultures containing about 10^9 cfu/ml 2 hr after saw-blade inoculation with *Penicillium digitatum* spores. Decay was assessed after 3 wk at 20 C. Different letters on columns within each color class indicate significant differences ($P \leq 0.05$) by Fisher's least significant difference test. Actual values are shown; statistical analysis used arcsine-transformed data.

Suppression of some foliar (29,31,32) and soilborne diseases has been reported (9,22,27).

Some potentially negative aspects of the pseudomonads need evaluation before commercial development can be recommended. *P. cepacia* can be a plant pathogen; it causes a soft rot of onion (37). Recently, one strain of *P. cepacia* was found to suppress wound healing and promote, rather than suppress, infection of orange fruit by *P. digitatum* (25). Although *P. cepacia* and *P. fluorescens* have not been associated with foodborne illness (21), some isolates are opportunistic colonizers of humans that are immunosuppressed or have other underlying disorders (17,46). The mammalian virulence of strains of *P. cepacia* from diverse origins has been studied. Strains from plants could be readily separated from those of clinical origin by the presence of pectolytic activity, the ability to macerate onion tissue, and bacteriocin production patterns (18). However, all strains were equally virulent to mice after intraperitoneal injection (18). *P. aeruginosa* is a significant human pathogen (46), whereas no isolations of *P. corrugata* from humans have been reported.

Antibiotic production by *P. cepacia* has been suggested to be the mode of disease control (23,26). Pyrrolnitrin is a broad-spectrum antifungal antibiotic (19,20). Many of its mammalian toxicological properties are known, and it has been used as a human medicine (19,20). The strain we tested produces between 5 and 7 μg of pyrrolnitrin per liter in 72 hr in nutrient broth (36). Immersion of inoculated lemons for 1 min in a minimum of 25–50 μg of pyrrolnitrin per liter controlled green mold (J. L. Smilanick, *unpublished*). Therefore, the failure of the cell-free culture fluid to control decay was expected. A role for pyrrolnitrin in the decay control we observed with pseudomonads is not supported because pyrrolnitrin-resistant isolates of *P. digitatum* were controlled by *P. cepacia*. Quantification of pyrrolnitrin in lemon wounds and the decay suppressiveness of *P. cepacia* isolates unable to produce pyrrolnitrin would help elucidate the role of this antibiotic. The growth of *P. cepacia* in wounds was very rapid. This growth presumably could deplete nutrients or deny space available to the pathogen and reduce the incidence of infections. Nutrient competition by pseudomonads was superior to many other epiphytes and inhibitory to the germination of the spores and growth of *Phoma betae* A. B. Frank and *Cladosporium herbarum* (Pers.:Fr.) Link on beet leaves (6).

P. corrugata was superior to *P. cepacia*

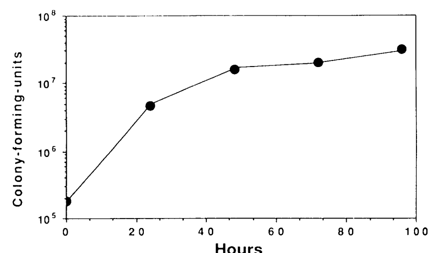

Fig. 3. Growth of *Pseudomonas cepacia* in 3 × 3 mm puncture wounds on lemons for 100 hr at 20 C.

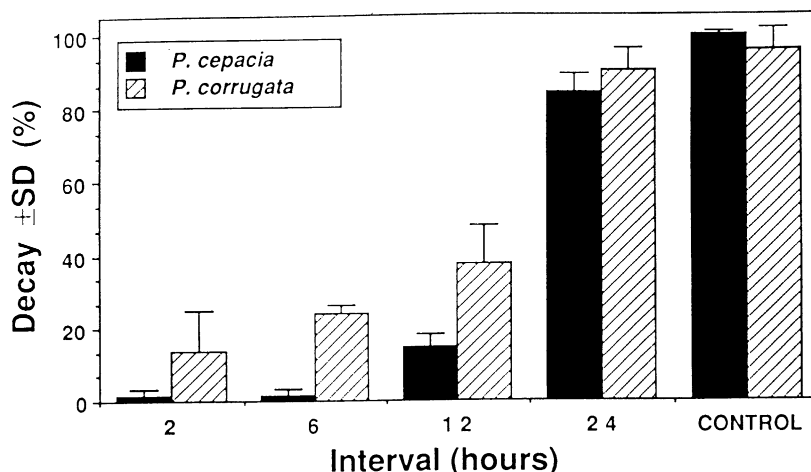

Fig. 4. Influence on postharvest decay of lemons of the interval between saw-blade inoculation with *Penicillium digitatum* and immersion of the fruit in water or bacterial cultures of *Pseudomonas cepacia* or *P. corrugata* containing about 10^9 cfu/ml. Decay was assessed after 7 days at 20 C. Controls present at each interval decayed more than 90%.

Table 1. The percentage of lemon fruit decayed by citrus green mold after inoculation of wounds with *Penicillium digitatum* followed 2 hr later with a 25-μl volume of water, 2.5 × 10^7 cfu of washed cells of *Pseudomonas* spp., or the cell-free culture fluids of 3-day-old *Pseudomonas* spp. cultures

Fraction	*P. cepacia*	*P. corrugata*	*P. aeruginosa*	*P. fluorescens*
Complete culture	0.0 a[z]	45.3 a	27.5 a	35.0 a
Washed cells	0.0 a	42.5 a	55.0 b	51.2 ab
Culture fluid	91.5 b	100.0 b	77.5 c	57.5 a–c
Water	98.7 b	100.0 b	100.0 d	71.3 c

[z] Decay was assessed after 1 wk at 20 C. Values followed by unlike letters within columns are significantly different ($P < 0.05$) by Fisher's least significant difference test. Actual values are shown; statistical analysis used arcsine-transformed data.

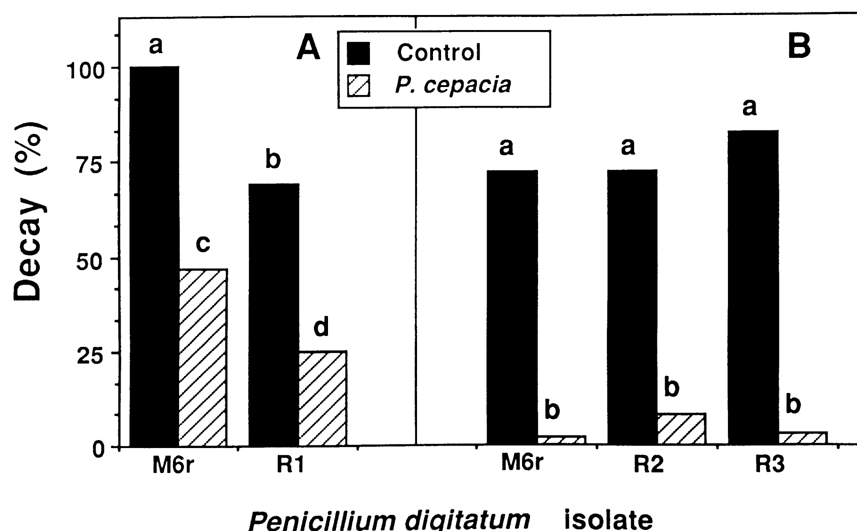

Fig. 5. Influence of pyrrolnitrin-resistance of *Penicillium digitatum* on its susceptibility to control by *Pseudomonas cepacia*. Lemons were immersed in *P. cepacia* cultures containing about 10^9 cfu/ml **(A)** 6 or **(B)** 2 hr after puncture inoculation with *P. digitatum* spores. *P. digitatum* isolate M6R is sensitive to pyrrolnitrin; isolates R1, R2, and R3 are resistant to pyrrolnitrin. Decay was assessed after 1 wk at 20 C. Different letters on columns indicate significant differences ($P \leq 0.05$) by Fisher's least significant difference test. Actual values are shown; statistical analysis used arcsine-transformed data.

in prior work with pseudomonads applied to peaches and nectarines to control postharvest brown rot, caused by *Monilinia fructicola* (G. Wint.) Honey, although *P. cepacia* could reduce decay significantly (40). In the present study, *P. cepacia* was consistently superior to *P. corrugata* for control of green mold in citrus. Biological control of citrus green mold offers several advantages over control of brown rot of stone fruit. Control of brown rot by the pseudomonads appears as a delay in disease onset (40), whereas with citrus green mold, the bacteria caused a reduction in disease incidence that persisted indefinitely. *M. fructicola* does not require wounds for infection, although infection is greatly facilitated by them (42), whereas *P. digitatum* requires a wound for infection to occur (16). Control of infections in wounds is more readily achieved, because bacteria can survive and colonize the potential infection courts. Decay from latent fungal inoculum, such as is characteristic of some infections of brown rot of stone fruit, has proven difficult to suppress (40). Moreover, potential hazards added bacteria pose to consumers can be minimized if the citrus rind is discarded before the fruit are consumed or processed, whereas ingestion of the bacteria cannot as easily be avoided with stone fruit. Finally, the growth of pseudomonads is facilitated by the warmer (10 C) storage used for citrus compared with the cold storage temperatures (0 C) used for stone fruit (16,42).

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