Promotion of Infection of Orange Fruit by *Penicillium digitatum* with a Strain of *Pseudomonas cepacia*

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ABSTRACT

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Susceptibility of wounds in orange fruit to infection by *Penicillium digitatum* decreased during periods of healing at 25 C and more so at 30 C. Healing was correlated with lignification in wounded tissue and phenylalanine ammonia-lyase (PAL) activity that was 0.2 units per milligram of protein per hour in fresh wounds and, after 72 h, was 5.9 units at 25 C and 8.8 units at 30 C. A strain of *Pseudomonas cepacia* (ID 2129), added to fresh wounds, suppressed the healing process as revealed by slower lignification, a PAL activity after 72 h of 1.1 units at 25 and 30 C, and a high susceptibility to infection by *P. digitatum*. The suppression was probably caused by a diffusible metabolite from *P. cepacia* present also in culture filtrates of the bacterium.

Additional keyword: wound healing.

Infection of citrus fruit by *Penicillium digitatum* (Pers.:Fr.) Sacc. occurs via wounds to the surfaces of the fruit (11) and can be promoted by additives that stimulate spore germination or germ-tube growth of the fungus (12). A period of healing of the wounds can minimize opportunities for infection, possibly through the synthesis of lignin and phenolic substances (3). During healing, phenylalanine ammonia-lyase (PAL) activity increases at sites of injuries (7).

Antagonistic yeasts and bacteria, including *Pseudomonas cepacia*, can be used to control infections of citrus fruit by *P. digitatum* (18), and also fungal infections of apple and pear fruit (9). In the course of assessing control of infections by *P. digitatum* on orange fruit by using *P. cepacia*, several closely related strains of this bacterium were obtained. One of them was found to promote fungal infections when it was applied to the injured fruit before the fungus was introduced. This promotion and its possible mode of action are reported here.

MATERIALS AND METHODS

Inocula. *P. digitatum* was isolated from decayed Washington navel oranges (*Citrus sinensis* (L.) Osbeck), and then cultured routinely on malt-extract agar (MEA) for 5 days at 25 C. Inocula for experiments were produced by wound-inoculating orange fruit, incubating the fruit at 25 C for 6 days in a humid plastic container, and then harvesting the conidia by brushing them into sterile distilled water containing 0.05% wetting agent Lisspol (ICI, Sydney, Australia). The suspension was filtered through four layers of muslin and adjusted to the required concentration.

Bacteria were isolated from the surface of Washington navel oranges harvested from the Somersby station of NSW Agriculture & Fisheries. Single colonies were obtained by streak-separating and subculturing on MEA. Organisms were identified from their fatty acid profiles on the Hewlett-Packard 5898A gas chromatography microbial identification system (Aerobe Library version 3.0, Microbial ID Inc., Newark, DE). Two isolates were thus identified as *P. cepacia*. One (ID 2131) isolate matched the library profiles very closely (0.888) and was inhibitive to growth of *P. digitatum* in vitro and in vivo (*unpublished data*). The other (ID 2129) isolate had a lower match (0.052) but was more closely related to *P. cepacia* than to any other species in the library

and was the promoter of infection by *P. digitatum* as described in this paper. This promoting isolate was routinely cultured on MEA for 5 days at 25 C. Bacterial cells were collected in sterile loops and placed in distilled water. The concentration of cells in suspension was determined as colony-forming units (cfu).

For production of culture filtrates, 50-ml aliquots of MEA broth (20 g of malt extract, 1 g of peptone, 20 g of dextrose, and distilled water [1:1]) in 250-ml conical flasks were inoculated with 1-ml suspensions of bacteria (1.9×10^{10} cfu/ml). After 72 h at 25 C on an orbital shaker (120 rpm), the broth was centrifuged at 15,000 *g* for 20 min. The supernatant was passed through a membrane (Millipore, 0.45 μm pore size) and used as culture filtrate. The precipitate was resuspended in distilled water and used as bacterial suspension.

Spore germination test. One-milliliter aliquots of spore suspensions of *P. digitatum* (7.15×10^5 conidia per milliliter) were added to 25-ml conical flasks together with 1-ml aliquots of distilled water or suspensions of *P. cepacia* (1.2×10^8 cfu/ml). These mixtures were then supplemented with 1-ml aliquots of fresh juice squeezed from orange fruit (50% in water) or of distilled water. Drops (50 μl) of each supplemental mixture were placed on separate, single new microscope slides, which were incubated at 25 C on glass frames above moist filter paper in petri dishes. After 15 and 20 h, 100 spores in each of six replicate drops per treatment were assessed for germination and germ-tube lengths, respectively.

Fruit inoculation. Experiments were done on mature orange fruit of the cultivars Washington navel, Bellamy navel, and Valencia. Fruit were harvested from the Somersby station of NSW Agriculture & Fisheries. Fruit were washed in tap water, sorted for uniform size, and inoculated by either of two methods. In the first method, each fruit was wounded to a depth of 1 or 3 mm at four equidistant points around the equator of the fruit. The wound was made with a sterile nail (2 mm in diameter), which protruded the required distance from a cork. In some experiments, the nail was dipped in inocula of *P. digitatum* between woundings. In other experiments, 20 μl of inocula of *P. digitatum* was pipetted into each wound made by the sterile nail. In the second method, wound sites were made at four equidistant points around the equator of the fruit by rubbing the surface with 60-grit sandpaper and causing a circular injury of 1 cm in diameter. These areas were immediately blotted on filter paper and then treated with 40-μl drops of inoculum.

After wounding, fruit were held at 25 or 30 C in plastic humidity chambers (>96% relative humidity [RH]). Depending on the experiment, inocula of *P. digitatum* were added immediately or up to 24 h after wounding, and were incubated under these conditions. Suspensions of *P. cepacia* were added at the time intervals shown in the tables.

Amounts of infection were measured by assessing the presence or absence of soft rot symptoms after 4 or 5 days at each of the four inoculation sites. The treatments were arranged in a complete randomized design. Each treatment was replicated twice with 10 fruit per replication. The experiment was repeated twice with similar results. Results were expressed as the percentage of inoculation sites showing symptoms, and data were transformed. Transformed data were analysed with an analysis of variance, and means were compared with Waller-Duncan's Bayesian *k*-ratio LSD rule ($k = 100$) (17).

Examination of lignin deposition and PAL activity. Bellamy navel and Valencia oranges were artificially injured by rubbing with 60-grit sandpaper and treated by pipetting a 40-μl cell suspension (1.2×10^9 cfu/ml) of *P. cepacia* onto each wound. Control fruit received the same amount of distilled water. Each treatment was replicated twice with 10 fruit for each replication. Fruit were then held in plastic bags (>96% RH) at 25 or 30 C.

Fruit were examined for lignin deposition by using toluidine blue O (14) and phloroglucinol-HCl (10). Segments were removed from injured sites and fixed in 95% ethanol for 16 h. Ten hand-sections were made from each segment and stained with either toluidine blue O or phloroglucinol-HCl. A total of 10 segments was examined per treatment.

PAL activity was measured by the methods previously described (7,15). Five grams of flavedo tissue from the injured area was removed and held in 100 ml of cold acetone at −15 C for 2 h. The acetone was changed twice during this period. After homogenizing in a Sorvall Omni-mixer for 5 min at the highest speed, homogenates were filtered through Whatman No. 1 papers, and the residues were washed with 100 ml of cold acetone. Residues from the filter paper were allowed to dry at room temperature for 1 h and then in a desiccator overnight at 4 C. Residues were stored at −15 C until assayed. The PAL assay was done following the method described by Chalutz (5), with 0.25 g of powdered residues in 10 ml of sodium borate buffer (0.1 M, pH 8.8). The concentration of cinnamic acid was measured at 269 nm on a Cary 2200 spectrophotometer. PAL activity was expressed as units per milligram of protein per hour. A unit of enzyme activity was defined as that amount of enzyme that catalyzed the conversion of 1 μmol cinnamic acid per hour. Protein was determined by the method described by Bradford (2). The experiment was repeated once.

RESULTS

Tests in vitro showed that the strain of *P. cepacia* did not promote germination and germ-tube growth of *P. digitatum* in water and decreased them in orange juice (Table 1).

Infection by *P. digitatum* was influenced by time between wounding and inoculation (Table 2). If fruit were wounded and inoculated immediately with the pathogen, decay reached a very high level regardless of whether *P. cepacia* or water was applied 24 h later. Delaying inoculation with *P. digitatum* for 10 or 24 h after wounding significantly reduced green mold decay in controls. There was no decline, however, in green mold decay after delays of inoculation with *P. digitatum* when *P. cepacia* had been added at the time of wounding. There was a significant interaction

TABLE 1. Effect of *Pseudomonas cepacia* on germination of *Penicillium digitatum* spores[y]

Treatment	15 h		20 h	
	Germination (%)	Germ-tube length (μm ± SE)	Germination (%)	Germ-tube length (μm ± SE)
Water	0 a[z]	0	0 a	0
Water + juice	65.2 d	73.7 ± 1.5	68.4 d	222.5 ± 3.8
P. cepacia	0 a	0	0 a	0
P. cepacia + juice	30.7 b	32.5 ± 1.3	49.3 c	82.5 ± 4.0

[y]One milliliter of spore suspension (7.15×10^5 spores per milliliter) of *P. digitatum* was mixed with an equal amount of distilled water or cell suspension (1.2×10^8 cfu/ml) of *P. cepacia*. These mixtures were then supplemented with 1 ml of fresh juice squeezed from orange fruit (50% in distilled water) or distilled water. Germination was assessed on 100 spores in each of six replicate drops per treatment, and germ-tube length was measured from germinated spores among them.

[z]Values in table followed by the same letter do not differ significantly by Waller-Duncan's Bayesian *k*-ratio LSD test ($k = 100$ level).

TABLE 2. Effect of treating wounds in Washington navel oranges infected by *Penicillium digitatum* with *Pseudomonas cepacia*

Treatment	Percentage of infection[y]			
	24 h earlier	Coincident	10 h later	24 h later
Water	90.0 ab[z]	85.2 b	65.1 c	58.5 c
P. cepacia	92.7 ab	85.4 b	98.7 a	99.4 a

[y]Fruit were wounded to a depth of 3 mm at four equidistant sites around the equator of the fruit, and then were inoculated at these sites with either 20 μl of water or *P. cepacia* (1.25×10^9 cfu/ml). Drops (20 μl) containing *P. digitatum* (3.6×10^5 spores per milliliter) were applied to wound sites at the time intervals relative to *P. cepacia* or water treatment shown above. Fruit were held in plastic bags at 25 C for 5 days.

[z]Values in table followed by the same letter do not differ significantly by Waller-Duncan's Bayesian *k*-ratio LSD test ($k = 100$ level).

between treatment and time ($P < 0.05$) with *P. cepacia*, resulting in high levels of infection at the later times.

When Washington navel oranges were injured at 1-mm depth and immediately inoculated with *P. digitatum* at any concentration, no significant infection developed (Fig. 1). Making the injury to 3-mm depth resulted in infection by *P. digitatum*. The infection at such a depth of injury increased linearly with increased concentration of *P. digitatum* above 3.6×10^3 spores per milliliter ($r = 0.95$). *P. cepacia* did not significantly affect green mold decay as compared with the control.

Temperature of wound healing significantly influenced infection by *P. digitatum* (Table 3) with a significant interaction between temperature and treatment ($P < 0.05$). Green mold decay was greatly reduced when Bellamy navel oranges were healed at 30 C for 3 days before being moved to 25 C, but much less so when cells or culture filtrates of *P. cepacia* were added at the time of wounding.

Green mold decay in fruit was significantly influenced by cultivar and cell concentration of *P. cepacia* (Table 4). A significant interaction between cultivar and treatment was detected ($P < 0.05$). Valencia was more resistant than Bellamy navel to infection by *P. digitatum* and only the highest concentration of *P. cepacia* overcame this resistance.

Host cell walls and the contents of collapsed cells in a layer three to four cells deep at the injured surface in control Valencia oranges were stained a blue-green color by toluidine blue O and a red color by phloroglucinol-HCl (Table 5). A response to staining with toluidine blue O in healed tissue was detected 24 h after injury, while a response to phloroglucinol-HCl staining occurred at 48 h after injury. No comparable staining could be detected 48 h after treatment with *P. cepacia* or its culture filtrate. An incomplete ring of cells responding to toluidine blue O, but not to phloroglucinol-HCl, was detected in some injured surfaces treated with *P. cepacia* or its filtrate at 72 h after injury.

PAL activity in Bellamy navel oranges at 30 C was significantly less 72 h after treatment with *P. cepacia* (1.31 ± 0.81 units per

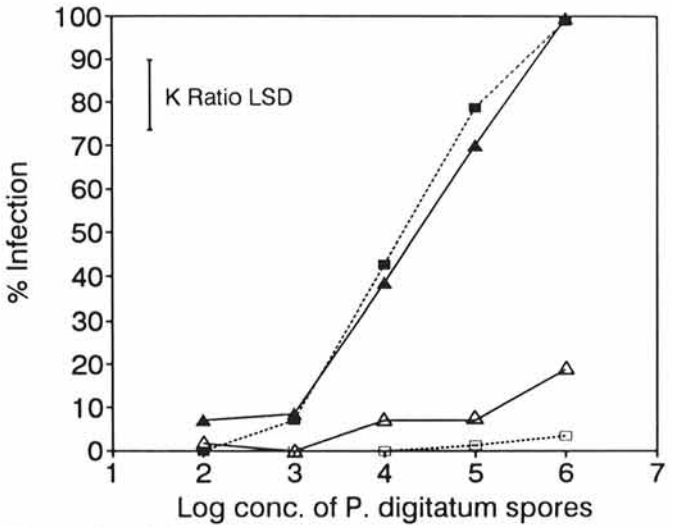

Fig. 1. Effect of immediate application of *Pseudomonas cepacia* (1.2×10^9 cfu/ml) on green mold development in Washington navel oranges. Fruit were inoculated at 1- or 3-mm depths with different concentrations of *Penicillium digitatum*. Water or *P. cepacia* (1.2×10^9 cfu/ml) was immediately applied to inoculated sites. Fruit were incubated at 25 C, >96% relative humidity, for 4 days. □ = Water + *P. digitatum* to a depth of 1 mm; △ = *P. cepacia* + *P. digitatum* to a depth of 1 mm; ■ = water + *P. digitatum* to a depth of 3 mm; ▲ = *P. cepacia* + *P. digitatum* to a depth of 3 mm. Vertical bar represents Waller-Duncan's Bayesian *k*-ratio LSD rule ($k = 100$ level).

TABLE 3. Effect of *Pseudomonas cepacia* or its culture filtrate applied 24 h after treatments to wounds infected by *Penicillium digitatum* on Bellamy navel oranges

Treatment applied at time of wounding	Percentage of infection[x]	
	25 C	30 C[y]
Water	61.3 c[z]	23.7 d
P. cepacia	89.1 a	71.3 b
Culture filtrate	87.4 a	71.3 b

[x] Fruit were wounded at four equidistant sites by rubbing 60-grit sandpaper (1 cm in diameter), and each wound was treated with 40 µl of *P. cepacia* (1.25×10^9 cfu/ml) or its culture filtrate. Fruit were then held in plastic bags at 25 C for 5 days.

[y] Fruits were held at 30 C for 3 days before being moved to 25 C.

[z] Values in tables followed by the same letter do not differ significantly by Waller-Duncan's Bayesian *k*-ratio LSD test ($k = 100$ level).

TABLE 4. Effect of orange cultivars and concentration of *Pseudomonas cepacia* on infection by *Penicillium digitatum*

Inoculum concentration (cfu/ml)	Percentage of infection[y]	
	Bellamy navel	Valencia
Water	61.3 c[z]	31.2 d
10^7	58.7 c	34.9 d
10^8	75.2 b	30.0 d
10^9	98.1 a	75.1 b

[y] Fruit were wounded with a nail to a depth of 3 mm at four equidistant sites around their equator; then fruit were treated with water or *P. cepacia* at different concentrations and were inoculated with *P. digitatum* (4.5×10^5 spores per milliliter) 24 h after treatment. Fruit were held in plastic bags for 5 days.

[z] Values in table followed by the same letter do not differ significantly by Waller-Duncan's Bayesian *k*-ratio LSD test ($k = 100$ level).

TABLE 5. Response of injured tissues of Valencia oranges to *Pseudomonas cepacia* and its culture filtrate at different stages during healing

Treatment	Responses of samples[z]					
	Toluidine blue O			Phloroglucinol-HCl		
	24 h	48 h	72 h	24 h	48 h	72 h
Water	+	+	+	−	±	+
P. cepacia	−	−	±	−	−	−
Filtrate	−	−	±	−	−	−

[z] + = Complete staining band with a blue-green color for toluidine blue O and a red color for phloroglucinol-HCl; − = no staining detected; ± = incomplete staining band at some wound sites.

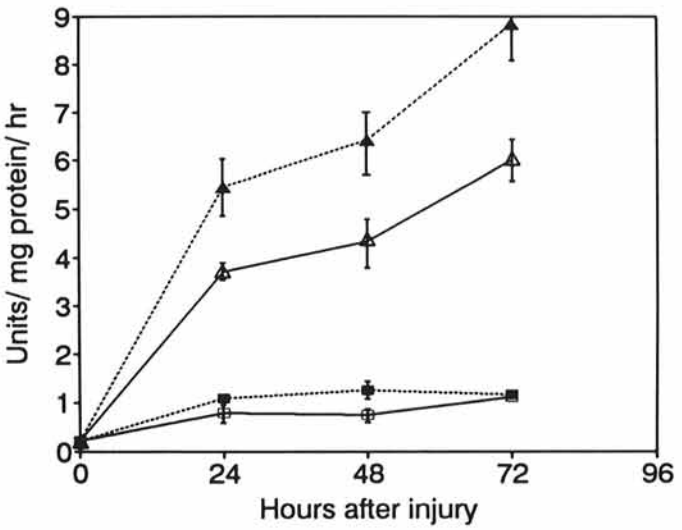

Fig. 2. Effect of *Pseudomonas cepacia* on phenylalanine ammonia-lyase activity at healing sites of wounded flavedo of Valencia oranges at 25 or 30 C. Water or *P. cepacia* (1.3×10^9 cfu/ml) was applied to wounds made with sandpaper. Fruit were then incubated at >96% relative humidity. △ = Water at 25 C; ▲ = water at 30 C; □ = *P. cepacia* at 25 C; ■ = *P. cepacia* at 30 C. Vertical bars represent the standard errors about the means of two replicates.

milligram of protein per hour) or its filtrate (1.40 ± 0.92) at the time of injury as compared with water controls (7.09 ± 1.31). A similar effect was shown in Valencia oranges (Fig. 2). The injured controls showed major increases in PAL activity during the healing period, particularly at 30 C. Application of *P. cepacia* at the time of injury prevented these increases.

DISCUSSION

A wound greater than 1 mm in depth in orange fruit is essential for infecton by *P. digitatum* (1,3). The wound can heal, thus minimizing the opportunities for infection at a later time (4,6,7). Healing is reported to include the activity of PAL and the synthesis of phenolic compounds (6–8, 16), corroborated here by the measurements of PAL and the positive staining for lignin-like materials (14) as tissues heal.

The healing process is easily disrupted by immediate inoculation with *P. digitatum* after injury (13). Delaying inoculation with P. digitatum, however, allowed the injured fruit to heal at 25 C and become less susceptible to *P. digitatum* infection than freshly injured tissue. Healing at 30 C was associated with enhanced PAL activity and greater resistance of injured tissue to infection by *P. digitatum*.

The strain of *P. cepacia* described here can promote infection by *P. digitatum* when the bacterium has been able to affect the wounded surfaces for some hours before the fungus is introduced. The mechanism of promotion is likely to be on the healing process in the fruit rather than by stimulation of the fungus, because of the lack of promoting effects on the germination of the fungus in vitro and of immediate effects in vivo. Evidence that the bacterium can suppress the increase in PAL and the acquisition of responsiveness to the stains for lignin-like materials as wounds heal supports this conclusion. Promotion of infection and suppression of healing also by culture filtrates of the bacterium indicate that a diffusible product(s) from the bacterium can inhibit the healing process either through a general action on metabolism in the fruit or by a particular action on aromatic biosynthesis.

An implication in minimizing postharvest loss is the need for vigilance particularly in considering the use of biocontrol agents such as strains of *P. cepacia* (9,18) for their effects on the healing process in fruit.

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