Postharvest Biological Control of Stone Fruit Brown Rot by *Bacillus subtilis*

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ABSTRACT

Pusey, P. L., and Wilson, C. L. 1984. Postharvest biological control of stone fruit brown rot by *Bacillus subtilis*. Plant Disease 68:753-756.

Pseudomonas cepacia, P. fluorescens, Bacillus thuringiensis, and two isolates of *B. subtilis* (B-3 and B-1849) were tested in the laboratory for antagonism against *Monilinia fructicola* on wounded stone fruit. Peaches, nectarines, apricots, and plums were sprayed with bacterial suspensions, inoculated 1–2 hr later with spores of *M. fructicola*, then held in moist chambers at 18–24 C. Only the B-3 strain of *B. subtilis* controlled brown rot on all fruit types. When varying concentrations of B-3 were tested on peaches, brown rot development was retarded by 10^6 and 10^7 colony-forming units per milliliter. At 10^8, fruit did not become infected with *M. fructicola* but decayed after 9 days because of other fungi. Strain B-3 reduced decay regardless of the inoculum level or the isolate of *M. fructicola* tested and was effective at temperatures of 10–30 C. The mechanism of B-3 activity appears to involve production of an antifungal substance, since the culture filtrate protected fruit from rot. The filtrate retained activity after being autoclaved at 15 psi and 120 C for 15 min.

Additional key words: bacterial antagonist

We depend primarily on chemicals to control brown rot, an important disease of *Prunus* spp. caused by *Monilinia fructicola* (Wint.) Honey. In recent years, widely used fungicides, such as benomyl and related benzimidazoles, have become much less effective because of the appearance of fungicide-resistant lines of *Monilinia* (21).

Biological control of brown rot has been suggested, although no effective attempts have been reported. In general, reports have been limited to observations of antagonism toward *Monilinia* on fruit (5,6,8,9) and microbial inhibition of *Monilinia* in vitro (1,5,8,9,12). Jenkins (8,9) described a method for isolating bacteria antagonistic to *M. fructicola* and reported a *Bacillus* species, probably *B. cereus*, associated with conidia on fruit in the orchard. It appeared to reduce the development of brown rot when introduced on the surface of mature fruit in the laboratory.

The purpose of our study was to investigate the potential of bacterial antagonists in the postharvest control of brown rot. Bacteria showing high antagonism toward *M. fructicola* in vitro were selected for the antagonism tests. Our results were previously reported in abstracts (13,14).

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Accepted for publication 12 March 1984.

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MATERIALS AND METHODS

The following bacteria were tested as antagonists: *Pseudomonas cepacia* Burkholder (Pc-41) and *P. fluorescens* Migula (Pf-21), obtained from Harvey Spurr, USDA-ARS, Tobacco Research Laboratory, Oxford, NC 27565; *Bacillus subtilis* (Cohn) Prazmowski (B-3), isolated at Kearneysville, WV; *B. subtilis* morphotype *globigii* (B-1849), obtained from William Haynes, USDA-ARS, Fermentation Laboratory, Peoria, IL 61604; and *B. thuringiensis* Berliner (HD-1), obtained from Howard T. Dulmage, USDA-ARS, Cotton Insects Research Laboratory, Brownsville, TX 78520. The B-3 isolate from Kearneysville is presumed to have come from either the soil or the surface of apple roots, since it appeared on a medium to which root sections had been added for the purpose of isolating pathogenic fungi. An isolate of *M. fructicola* (WV-20), collected from a peach orchard, was used as inoculum.

Test fruit were from orchard trees at the Appalachian Fruit Research Station. During fruit development, trees had been sprayed only with captan as a fungicide at 5.6 kg per hectare. Before being tested in the laboratory, fruit were washed in water with 0.05–0.2% Tween 20. Nectarine, apricot, and plum clones used were Harko, V66022, and BY68-87, respectively. Peach clones used, depending on availability, were LaGem, Norman, Loring, Mountain Gold, and B70446. Fruit were collected at the stage of maturity when fruit are normally harvested commercially for storage or transit.

Antagonism tests. In vitro tests were performed on potato-dextrose agar (PDA) and nutrient-yeast-dextrose agar (NYDA; 8 g of nutrient broth, 5 g of yeast, 10 g of dextrose, and 15 g of agar per liter of medium) in 9-cm petri plates. Plates were streaked across the center with the bacterium, incubated at 25 C for 2 days, then sprayed with a 10^5 spores per milliliter aqueous suspension of *M. fructicola*. After 3 days of additional incubation, width of the inhibition zone was measured for each of six plates per strain.

All five bacterial strains were applied in suspension to wounded surfaces of peaches, nectarines, apricots, and plums, and fruit were subsequently sprayed with spores of *M. fructicola*. Bacteria were initially transferred from nutrient agar slants (stored at 5 C) to 50 ml of nutrient-yeast-dextrose broth (NYDB; 8 g of nutrient broth, 5 g of yeast, and 10 g of dextrose per liter) in 250-ml flasks. After incubation on a gyratory shaker at 200 rpm and 30 C for 24 hr, 5 ml of the liquid culture was transferred to 1-L Erlenmeyer flasks containing 250 ml of NYDB. After 3 days at 200 rpm and 30 C, bacterial cells were harvested by centrifugation at 6,000 $\times g$ for 20 min, then resuspended in water.

Fruit were wounded with a dissecting knife that had been forced through a cork stopper so the protruding point would make a wound approximately 3 mm in diameter and 3 mm deep. Ten fruit per treatment were sprayed to runoff with bacterial suspensions at 10^7 colony-forming units (CFU) per milliliter; control fruit were sprayed with water. A 600-ppm benomyl treatment was included for comparison. After drying for 1–2 hr, fruit surfaces were spray-inoculated with a 10^5 spores per milliliter aqueous suspension of *M. fructicola*. The fruit were then held at 18–24 C in moist chambers. After 2 days for plums and 3 days for all other fruit, treatments were compared based on diameter of decay lesions. Various concentrations of bacteria (10^5–10^9 CFU per milliliter) were later tested on peach fruit against 10^5

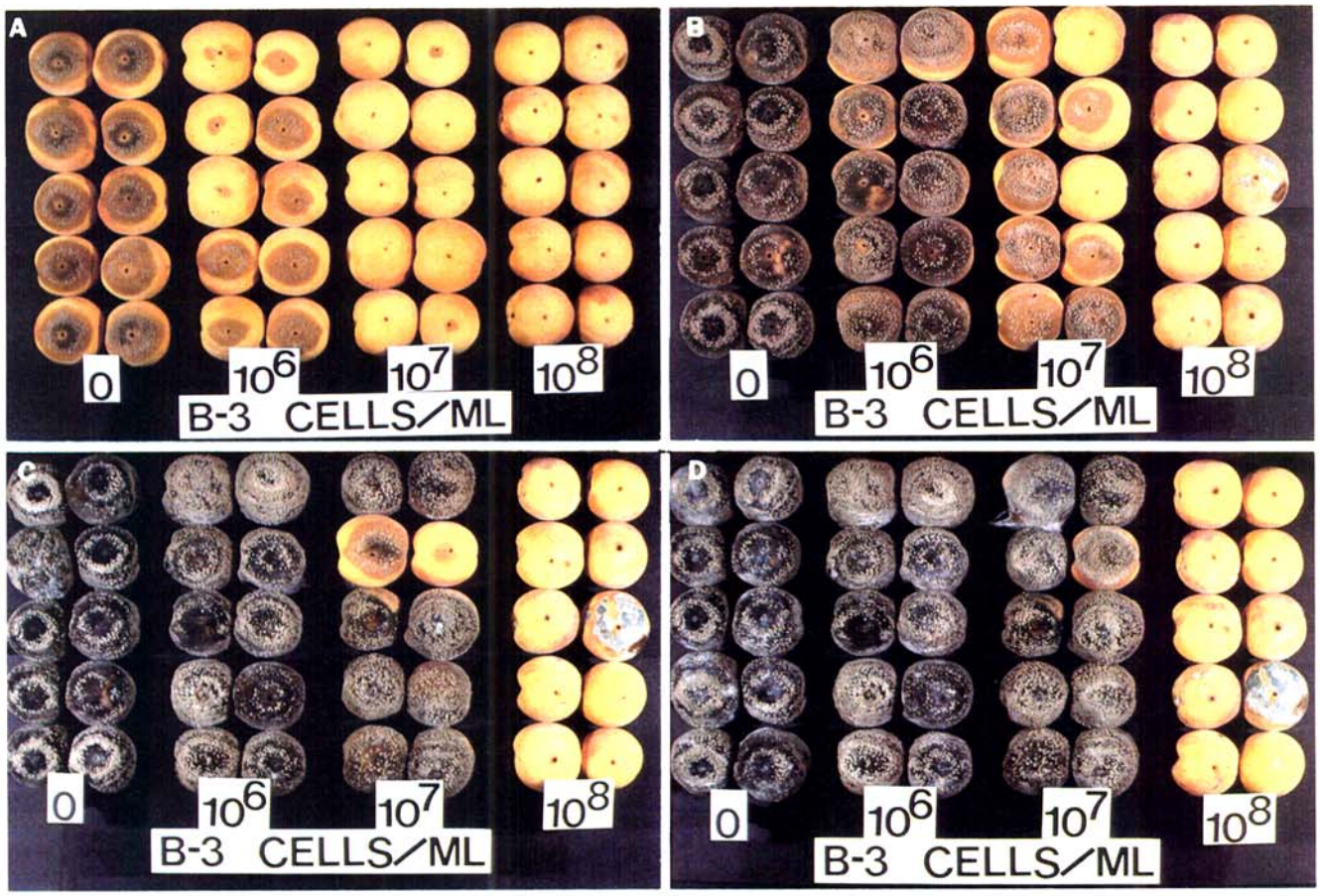

Fig. 1. Progression of brown rot development over 3–9 days on peaches treated with varying concentrations of *Bacillus subtilis* (B-3): **(A)** Three days after inoculation, 10^6 CFU per milliliter of B-3 partially reduced decay. **(B)** After 5 days, all but two peaches sprayed with 10^7 CFU per milliliter of B-3 were rotted, and **(C)** these showed decay after 7 days. **(D)** Even after 9 days, none of the fruit treated with 10^8 CFU per milliliter of B-3 were infected with *Monilinia*. Blue-green growth is that of *Penicillium expansum*, which appeared to initiate infection at a natural wound site.

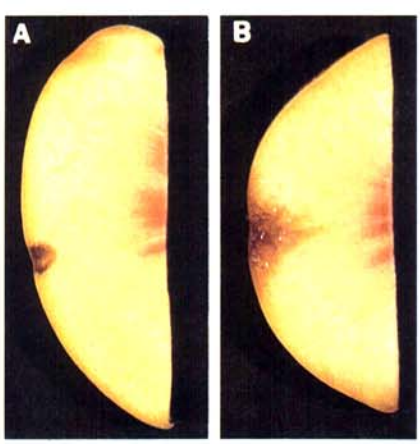

Fig. 2. Peach sections 2 days after wounding and treatment with *Bacillus subtilis* (B-3): **(A)** Tissue lining wound cavity of treated fruit is darkened. **(B)** Discoloration associated with infection by *Monilinia fructicola* extends to internal tissue of control fruit.

fungal spores per milliliter.

Procedures for subsequent tests with *B. subtilis* were the same as described, except where specified. Strain B-3 was challenged with 10^3–10^7 spores per milliliter of *M. fructicola*. It was also tested against four other isolates of *M. fructicola* (at 10^5 spores per milliliter) from different areas of the United States (California, Missouri, New York, and South Carolina). The effect of temperature on B-3 antagonism was studied by holding fruit in moist chambers at 5, 10, 15, 20, 25, and 30 C after treatment and inoculation.

Antibiotic activity. The possible involvement of antibiotic production in B-3 antagonism was tested as follows. The B-3 cells were separated from the culture medium by one centrifugation cycle at 6,000 × *g* for 20 min as previously, but a portion of the B-3 cells was resuspended in water and centrifuged a second time to wash cells and further remove any extracellular toxins. After cells were suspended again in water, a sample of the suspension with washed cells was autoclaved at 15 psi and 120 C for 15 min. The supernatant medium from the first centrifugation cycle was passed through a 0.3-μm pore membrane filter to eliminate any residual cells, and a sample of the filtrate was autoclaved for 15 min. Fruit were treated with cells collected by centrifuging once, cells washed by centrifuging twice, cells killed by autoclaving, the cell-free culture filtrate, or the autoclaved culture filtrate. In addition to a water control, fruit were treated with fresh NYDB medium. All fruit were inoculated as previously described.

RESULTS

All five bacterial strains inhibited radial growth of *M. fructicola* on PDA and NYDA (Table 1), with the widest zones of inhibition resulting from antagonism by the two *Pseudomonas* spp. These bacteria, however, had little or no effect on the pathogen in vivo (Table 2). Significant differences between *Pseudomonas*-treated fruit and control fruit were detected only with nectarines. The B-1849 strain of *B. subtilis* reduced decay of peaches, nectarines, and plums, whereas *B. thuringiensis* reduced decay of plums only. The B-3 strain of *B. subtilis* had a dramatic effect on brown rot development on all fruit types. Strain B-3 prevented brown rot of peaches and apricots after 3 days and substantially reduced decay of plums and nectarines after 2 and 3 days, respectively. Benomyl prevented decay of all fruit.

Pseudomonas spp. had minimal effects on decay of peach fruit even when concentrations were increased to 10^9 CFU per milliliter. For strains B-1849 and B-3 of *B. subtilis*, suspensions of adequate volume with concentrations higher than 10^7 and 10^8 CFU per milliliter, respectively, were not obtained with the growth conditions and volume of medium used. Three days after inoculation, 10^6 CFU per milliliter of B-3 had partially

reduced peach decay, 10^7 had prevented decay of most fruit, and 10^8 had prevented decay of all fruit (Fig. 1). After 5 days, all but two peaches sprayed with 10^7 CFU per milliliter were rotted. After 9 days, none of the peaches treated with 10^8 CFU per milliliter had become infected with *M. fructicola* but had succumbed to other fungi, such as *Penicillium expansum* and *Rhizopus stolonifer*.

Although fruit of different peach cultivars were used in different antagonism tests, reaction to the antagonist/pathogen challenge appeared to be the same. Only tissue lining the wound cavity of treated fruit was discolored, but internal decay of untreated fruit was apparent after 1 or 2 days (Fig. 2). Fruit were rot-free 3 days after treatment with 10^8 CFU per milliliter of B-3 regardless of the isolate or the inoculum level of *M. fructicola* used. When fruit were held at temperatures of 5–30 C after treatment with B-3 at 10^7 CFU per milliliter, the rate of decay for the control fruit increased with temperature (Table 3). Very little decay of treated fruit was observed after 5 days at any one temperature, however.

The cell-free filtrate from the B-3 culture protected fruit from *M. fructicola* to the same extent as B-3 suspensions of 10^7 CFU per milliliter in water (Table 4). The protective ability of B-3 was decreased by washing the cells and completely destroyed by autoclaving the suspension. The activity of the culture filtrate was only partially lost by autoclaving. Fruit treated with NYDB decayed at a greater rate than control fruit sprayed with water.

DISCUSSION

Although the *Pseudomonas* strains were highly antagonistic to *M. fructicola* on agar media, these organisms showed minimal or no antagonism on the fruit surface. Possibly, the bacteria survived poorly on fruit, cells from liquid culture did not have the same ability as cells grown on solid media to produce inhibitory substances, or inhibitors produced by the bacteria were unstable or inactive in the fruit surface environment.

B. thuringiensis did not perform well on fruit despite previous encouraging results (13). With the *B. subtilis* strains, some antagonism by B-1849 in vivo was indicated, but antagonism by B-3 on fruit was eminent.

Biocontrol of brown rot with B-3 was more effective on peaches and apricots than on nectarines and plums. Perhaps this is related to basic differences in the fruit surfaces. One type is pubescent and the other is smooth and waxy. The hairs may improve adherence of bacteria to fruit or increase the area or number of sites on which cells may reside.

Benomyl was more effective than B-3 against brown rot. Neither benomyl nor B-3 was observed to affect natural infection by *R. stolonifer*. Just as benomyl is frequently combined with dicloran (Botran) to control Rhizopus rot, possibly an antagonist such as B-3 could be mixed with a chemical fungicide or another antagonist to control this other important disease.

It is possible that not all the antifungal activity on fruit is due to bacterial growth and antibiotic production. Considering that washed cells were less effective (Table 4), some inhibition could result from a carry-over of toxic compounds, such as NH_3, accumulated in the culture medium. On the other hand, a low molecular weight polar metabolite showing biological activity against *M. fructicola* in vitro was isolated from the culture filtrate (C. C. Reilly and P. L. Pusey, *unpublished*). It could be autoclaved at an acid or neutral pH but lost activity at a pH between 8 and 10. Heat-stable antifungal substances produced by *B. subtilis* have been reported previously (2,10,12,15,19).

Increasing the B-3 concentration from 10^6 to 10^7 CFU per milliliter improved biocontrol, and a further increase to 10^8 totally controlled brown rot. Higher B-3 concentrations extended the period before decay was initiated by *Monilinia*. Once started, however, lesions on fruit with different concentration treatments seemed to enlarge at the same rate. Apparently, the mode of B-3 activity on fruit is toward spore germination or early

Table 1. Bacterial inhibition of *Monilinia fructicola* in vitro[x]

Bacterium	Inhibition zone (mm)[y]	
	PDA	NYDA
Pseudomonas fluorescens (Pf-21)	27 a[z]	17 b
P. cepacia (Pc-41)	22 b	20 a
Bacillus subtilis (B-3)	16 c	16 bc
B. subtilis (B-1849)	14 c	15 c
B. thuringiensis (HD-1)	5 d	6 d

[x] Plates were streaked across center with bacterium, sprayed with spore suspension of *M. fructicola* after 2 days at 25 C, then evaluated after 3 days of incubation.
[y] Each value is the mean of six plates. PDA = potato-dextrose agar; NYDA = nutrient-yeast-dextrose agar.
[z] Means in a column followed by the same letter do not differ significantly at $P = 0.05$ by Duncan's multiple range test.

Table 2. Postharvest reduction of brown rot of stone fruit by antagonistic bacteria[x]

Treatment	Diameter of decay lesions (mm)[y]			
	Peaches	Nectarines	Apricots	Plums
Bacillus subtilis (B-3)	0 c[z]	7 c	0 c	4 d
B. subtilis (B-1849)	20 b	38 b	29 b	27 c
B. thuringiensis (HD-1)	37 a	43 ab	39 a	28 bc
Pseudomonas cepacia (Pc-41)	41 a	41 b	39 a	30 ab
P. fluorescens (Pf-21)	38 a	42 b	36 ab	29 ab
Benomyl	0 c	0 d	0 c	0 e
Control	41 a	48 a	35 ab	31 a

[x] Fruit were wounded, treated, and artificially inoculated, then incubated at 18–24 C in moist chambers; plums were evaluated after 2 days and all other fruit after 3 days.
[y] Each value is the mean of 10 fruit.
[z] Means in a column followed by the same letter do not differ significantly at $P = 0.05$ by Duncan's multiple range test.

Table 3. Effect of temperature on antagonism of *Bacillus subtilis* (B-3) to *Monilinia fructicola* on peach fruit[y]

Temperature (C)	Diameter of decay lesions (mm)[z]	
	B-3	Control
5	0	0
10	0	2
15	1	25
20	1	63
25	4	73
30	0	78

[y] Fruit were wounded, treated, and artificially inoculated, then incubated in moist chambers for 5 days.
[z] Each value is the mean of 10 fruit. Means of treated and control fruit were significantly different ($P = 0.05$) at temperatures of 10 C and above.

Table 4. Effect of *Bacillus subtilis* (B-3) cells suspended in water and cell-free culture filtrate on brown rot of peach[w]

Treatment[x]	Diameter of decay lesions (mm)[y]
B-3	0 d[z]
B-3 (washed)	9 c
B-3 (autoclaved)	30 b
Culture filtrate	0 d
Culture filtrate (autoclaved)	9 c
NYDB	36 a
Water	30 b

[w] Fruit were wounded, treated, and artificially inoculated, then incubated at 18–24 C in moist chambers for 3 days.
[x] B-3 suspensions consisted of cells collected by one centrifugation cycle, washed by centrifuging twice, or killed by autoclaving. Culture filtrates were obtained by passing supernatant through a 0.3-μm pore filter. NYDB = nutrient-yeast-dextrose broth.
[y] Each value is the mean of 10 fruit.
[z] Means in a column followed by the same letter do not differ significantly at $P = 0.05$ by Duncan's multiple range test.

germ tube development, with minimal effect on subsequent fungal growth.

Antagonism of B-3 on fruit appeared to be functional at all temperatures that permitted *Monilinia* to decay untreated fruit. Assuming that activity is due largely to B-3 growth and antibiotic production, our results are in contrast to those of another study in which wheat seed was treated with *Bacillus* spp. to protect against soil fungi (11). The fungi grew at temperatures lower than the minimum temperature required for bacterial growth or antibiotic production. The effect of temperature on B-3 performance is of importance because fruit may be subjected to a wide temperature range between time of treatment in the packing plant and time of use by the consumer.

Biological control has been demonstrated less for pathogens attacking aerial parts of plants than for those in the soil (3), and only a few examples can be cited for biocontrol of pathogens that directly affect the fruit (16–18,20). Possibly, microorganisms antagonistic to pathogens of the mature fruit or plant product have a greater potential for disease control after harvest. Many uncontrollable variables exist under the field situation, but the postharvest environment can be more easily manipulated to favor the antagonist. Biocontrol in the stored-product environment is newer to the pathologist than to the entomologist (4,7) dealing with postharvest pest problems. Our results show the potential for biocontrol of postharvest decay. This potential might even extend to direct use in the market to prolong shelf life, provided the antagonist and its metabolites are safe for human consumption.

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