Pilot Tests for Commercial Production and Application of *Bacillus subtilis* (B-3) for Postharvest Control of Peach Brown Rot

P. L. PUSEY, Research Plant Pathologist, and M. W. HOTCHKISS, Research Technologist, USDA-ARS, Southeastern Fruit and Tree Nut Research Laboratory, P.O. Box 87, Byron, GA 31008; H. T. DULMAGE, Research Microbiologist, USDA-ARS, Subtropical Crops Insects Research, P.O. Box 1033, Brownsville, TX 78520; R. A. BAUMGARDNER, Professor, Department of Horticulture, and E. I. ZEHR, Department of Plant Pathology and Physiology, Clemson University, Clemson, SC 29631; C. C. REILLY, Research Plant Pathologist, USDA-ARS, Southeastern Fruit and Tree Nut Research Laboratory; and C. L. WILSON, Research Plant Pathologist, USDA-ARS, Appalachian Fruit Research Station, Route 2, Box 45, Kearneysville, WV 25430

ABSTRACT

Pusey, P. L., Hotchkiss, M. W., Dulmage, H. T., Baumgardner, R. A., Zehr, E. I., Reilly, C. C., and Wilson, C. L. 1988. Pilot tests for commercial production and application of *Bacillus subtilis* (B-3) for postharvest control of peach brown rot. Plant Disease 72:622-626.

The potential of *Bacillus subtilis* for postharvest control of peach brown rot caused by *Monilinia fructicola* was assessed in pilot tests on simulated commercial packing lines at Byron, GA, and Clemson, SC, and in a commercial packinghouse in Musella, GA. Because disease pressure was low due to drought conditions (1986), inoculum was introduced either in the dump tank water or as a spray onto the fruit after treatment on the line. Strain B-3 of *B. subtilis* was cultured in nutrient-yeast-dextrose broth (NYDB) in flasks at Byron or in another medium contained in a 250-L fermentor at Brownsville, TX. Nutrient broth was replaced by cottonseed flour as the primary source of nitrogen in the fermentor. This medium was low in cost, suitable for commercial use, and yet supported antibiotic production in culture, as did NYDB. In packing line tests, the number of colony-forming units of the bacterium added to each fruit was between 2×10^7 and 7×10^7 for B-3 from flask cultures and approximately 2×10^9 for B-3 from the fermentor culture. In a test at Byron, B-3, cultured in flasks and applied fresh or after being stored (as paste or powder), was equal to benomyl (1–2 ppm residue in fruit) for brown rot control. In a similar test at Clemson, stored forms of B-3 from both flask and fermentor cultures were as effective as benomyl. In the packinghouse, B-3 produced at Byron approached benomyl for disease control, whereas that produced in the fermentor had no effect. However, an inability to completely remove fungicide residues from equipment in the packinghouse and the need to spray-inoculate fruit twice with the pathogen introduced unusual circumstances.

Biological control may be an alternative to chemical control of brown rot of stone fruit caused by *Monilinia fructicola* (Wint.) Honey. In recent years, chemical fungicides have become less effective due to the development of pathogen resistance (3,7,11,13). Also, a heightened public awareness of pesticide residues in food and the possibility of greater restrictions in the use of chemicals on harvested crops are bases for considering alternatives. The potential of postharvest biological control of plant diseases has been discussed (12).

In previous studies, an antibiotic-producing strain of *Bacillus subtilis* (Ehrenberg) Cohn (2,6) reduced brown rot when wounded fruit were treated with *B. subtilis* and subsequently challenged with spores of *M. fructicola* (8). Subsequently, *B. subtilis* was found compatible with commercial fruit waxes and with dicloran, a fungicide commonly used after harvest for *Rhizopus* control (9). In the following report, *B. subtilis* was formulated with dicloran and fruit wax and tested on simulated and actual commercial peach-packing lines.

Accepted for publication 8 February 1988.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1988.

MATERIALS AND METHODS

Experiments were conducted in 1986 on simulated peach-packing lines at the USDA-ARS, Southeastern Fruit and Tree Nut Research Laboratory, Byron, GA, and Clemson University, Clemson, SC. An additional test was performed in a commercial packinghouse at Dickey Farms, Musella, GA, using equipment presently used for chemical fungicide application.

Cultures. The B-3 strain of *B. subtilis*, originally collected in 1981 from the rhizosphere of apple in Kearneysville, WV, was used in all tests. The organism was stored on silica gel at −20 C (10). Cultures used for the experiments were grown in 2-L flasks at Byron, or, to simulate commercial production, in an IF-250 fermentor (250-L volume; New Brunswick Scientific Co., Edison, NJ) at the USDA-ARS, Subtropical Crop Insects Research Unit, Brownsville, TX.

At Byron, B-3 was started on nutrient-yeast-dextrose agar (NYDA) (8 g of BBL nutrient broth, 5 g of Difco yeast extract, 10 g of dextrose, and 15 g of Difco agar per liter of water) and then transferred to 50 ml of nutrient-yeast-dextrose broth (NYDB) (same ingredients as NYDA, without agar) in 250-ml flasks. The flasks were clamped on a rotary shaker and agitated at 180 rpm for 24 hr at 30 C. Next, the culture was transferred to 500 ml of NYDB in 2-L flasks that were shaken at 180 rpm. After 72 hr, the B-3 concentration was approximately 5.0×10^7 colony-forming units (cfu) per milliliter, and total solids in the cultures amounted to approximately 2.4%. These cultures were concentrated using a Pellicon Cassette filtration system (Millipore Corp., Bedford, MA) with a 100,000 molecular weight filter cassette. The concentrated culture was centrifuged at 5,500 *g* for 20 min. Pelleted material was used immediately, stored as a wet paste at 4 C, or dried in an oven at 35 C for 24 hr and then stored at 4 C. Conversion of fresh cultures to stable wet or dry forms resulted in approximately a 76% reduction in total cfu.

Before culturing B-3 in the 250-L fermentor at Brownsville, media suitable for commercial use were tested using Microferm bench-top fermentors (New Brunswick Scientific Co.) equipped with 14-L vessels. The media tested consisted of Proflo (partially defatted cooked cottonseed flour; Traders Protein Division of Traders Oil Mill Co., Fort Worth, TX) or Hy-Soy (defatted soybean meal; Sheffield Products, Memphis, TN) as the primary source of nitrogen. These materials, used successfully in fermentation media for *Bacillus thuringiensis* Berliner (1), were preferred in place of nutrient broth because of their lower cost. The bacterium was streaked on solid NYDA in petri plates. After 48 hr, 5 ml of sterile deionized water was added to the plates and the bacterial colonies were gently rubbed with a bent glass rod to make a suspension. A 0.5-ml sample of the suspension was used to inoculate 675 ml of NYDB in a 2-L flask. The flask was shaken at 340 rpm for 6–8 hr at 30 C. A 50-ml sample from the culture was then added to 10 L of the test medium in a 14-L fermentor. Fermentation conditions were: aeration, 0.666 v air/v medium/min;

agitation, 500 rpm; temperature, 30 C. Due to the vigorous and persistent foaming associated with the culture, an antifoam agent (25% Mazer DF 271 SX) was automatically fed into the system as needed. Culture samples were withdrawn after 36 and 60 hr. The antibiotic produced by B-3 was both extracted and tested against *M. fructicola* using the methods of McKeen et al (6). The crude extract was placed in wells made in an asparagine-dextrose agar medium previously seeded with spores of the fungus. The only modification of McKeen et al was the addition of 25 mg/ml of streptomycin sulfate to the medium to inhibit bacterial growth. The extract was also tested against *M. fructicola* on Chilean nectarines obtained from a grocery store in April. Ten fruit per treatment were wounded (8), treated with 30 μl of the extract, and then, 45 min later, the wounds were inoculated with 20 μl of a 10^5 spores/ml suspension of *M. fructicola*. After 6 days at 25 C, the diameter of lesions was measured.

Based on the above results, a medium was selected for use in the 250-L fermentor. It consisted of 15 g of Proflo, 30 g of dextrose, 5 g of yeast, 5 g of peptone, 1.0 g of $CaCO_3$, 0.3 g of $MgSO_4 \cdot 7H_2O$, 20 mg of $FeSO_4 \cdot 7H_2O$, 20 mg of $ZnSO_4 \cdot 7H_2O$, and 20 mg of $MnSO_4 \cdot H_2O$ per liter of water. It was adjusted to a pH of 7.0 and sterilized, with agitation, for 25 min at 121 C and 15 psi. Strain B-3 was started as described above except that the entire culture in the 2-L flask (675 ml) was added to the growth medium (125 L) in the 250-L fermentor. Fermentation conditions were the same as for the 14-L fermentor except that agitation was at 250 rpm rather than 500 rpm. After 60 hr, pH of the culture was reduced to 7.0 with 2 N HCl. The culture, which consisted of 6.5% solids, was harvested using a Sharples AS-16V Super centrifuge, continuous flow with a clarifying bowl. This procedure resulted in the conversion of each milliliter of the fresh culture suspension to 3.5×10^9 cfu of stabilized B-3 in the form of a paste. One-half of the paste was placed in a spray-drying apparatus to give 3.1×10^9 cfu of B-3 in the dry form for every 1 ml of starting culture. Both wet and dry forms of harvested B-3 were stored at 4 C.

Packing line equipment. At Byron, fruit were placed into a 475-L circulating dump tank filled with water. Elevating rollers lifted the fruit to a washer unit (Durand-Wayland Inc., La Grange, GA) that consisted of nylon brushes for washing fruit and foam rubber rollers for drying. Fruit then moved through a waxing unit (Decco Tiltbelt, Pennwalt Corp., Monrovia, CA) that consisted of a nylon brush bed and two side-by-side nozzles (set-up no. 22B for 1/4 J round spray; Spraying Systems Co., Wheaton, IL) for wax application. The packing line bed width was reduced to 30 cm with wooden rails.

At Clemson, all basic packing line equipment used was built by Durand-Wayland, Inc. Fruit moved from a 3,060-L circulating dump tank filled with water to a washer/dryer unit similar to the one described above, and then through a waxer with two overhead nozzles (set-up no. 26 for 1/4 J round spray, Spray Systems Co.) mounted in succession. The bed width was reduced to 22 cm with wood rails. Fruit moved through the system at the rate of approximately 13.6 kg/min and test material was applied at approximately 10 ml per kilogram of fruit.

At the packinghouse in Musella, GA, fruit from a commercial orchard were culled by hand, hydrocooled in bins, and then stored in a cold room (2–4 C) for 2 days before the test. The 91-cm-wide packing line (FMC Corp., Woodstock, VA) included a dry dump conveyor, a nylon brush washer, foam rubber rollers for drying, and a waxer with an overhead single nozzle that continually moved from side to side. Since the wax and fungicide spray did not hit all peaches passing through the waxer, adequate dispersal to all fruit was dependent on nylon brushes being saturated with the material. For all systems described above, fruit were transferred by hand from a conveyor at the end of the packing line to deep pocket fiber trays (no. 100; Keyes Fibre Company, Stamford, CT) that were stacked in 1-bu cardboard boxes.

Residue analysis. The simulated commercial systems were calibrated to attain recommended fungicide residue levels for fruit of approximately 2–3 ppm of dicloran and 1–2 ppm of benomyl. Rates were varied by altering concentration and flow rate. Fruit were then analyzed for residue. Although fruit from the packinghouse at Musella were also analyzed, spray equipment was not altered and fungicides were applied at approximately the same concentrations (5.25 mg/ml of dicloran and 1.75 mg/ml of benomyl) normally used by the packer. Only once were fruit analyzed for both dicloran and benomyl. Afterwards, benomyl residues were estimated based on the simpler dicloran analysis.

From each replicate in a test treatment, three or four whole fruit with a weight of 350–400 g were analyzed. The pulp was placed in a blender with water equal to 50% of the pulp weight and homogenized for 1 min. The homogenate was used for both dicloran and benomyl residue analyses.

Analysis for dicloran residue was performed using a modification of the procedure of Kilgore et al (4). One-hundred grams of the fruit homogenate was transferred to a 250-ml flask. Fifty milliliters of isooctane were added and the flask was stoppered and placed on a rotary shaker at 180 rpm for 15 min. When phases separated, 3 ml of the organic phase was withdrawn with a pipette and transferred to a vial containing 2–3 g of anhydrous sodium sulfate. Dicloran concentrations were determined with a Hewlett Packard 5890 gas chromatograph equipped with a 5 m $\times$ 530 μm methyl silicone column and an electron capture detector. Chromatography parameters included: oven, 160 C; injector, 200 C; detector, 300 C; nitrogen flow, 25 ml/min.

Benomyl residue analysis was done using a modification of Kirkland et al (5) which involved extraction of the active breakdown product, methyl 2-benzimidazol. Fifty grams of the homogenate was transferred to a 250-ml flask. Two milliliters of 2.6 N NaOH and 100 ml of ethyl acetate were added. The flask was placed on a rotary shaker at 180 rpm for 10 min and the ethyl acetate was decanted into a 500-ml round bottom flask. Extraction of the homogenate with 100 ml of ethyl acetate was repeated twice. Twenty milliliters of 1.0 N HCl was added and the sample was placed on a Buchi Rotavapor R110 (Brinkman Instruments, Westbury, NY) at 50 C for 15–20 min. The residual extract was then washed from the flask with 10 ml of hot water, three times with 50 ml of hexane, and once with 30 ml of ethyl acetate. The organic phase was removed with the use of a 250-ml separatory funnel and 6.5 N NaOH was added to the aqueous phase. The sample was extracted in the separatory funnel three times with 75 ml of ethyl acetate and then passed through 5–10 g of anhydrous sodium sulfate on filter paper. The extract was placed in a 500-ml round bottom flask and reduced to 15–20 ml with the Rotavapor R110. One milliliter of 0.1 N phosphoric acid was added and the extract was further reduced by evaporation in a 50-ml pear-shaped flask to approximately 1 ml. The sample was placed in a 2-ml volumetric flask and diluted to 2 ml with 0.1 N phosphoric acid. It was then injected into an ODS C-18 column in a Waters high pressure liquid chromatograph system (Waters Associates, Inc., Milford, MA). The mobile phase consisted of 450 ml of water, 0.5 g of sodium acetate, 50 ml of acetic acid, and 500 ml of methanol. Parameters included 300 psi at 1 ml/min and detection at 280 nm.

Fruit treatments. Cultivars used at Byron, Clemson, and Musella, were Redhaven, Redglobe, and Blake, respectively. Sulfur and/or captan were applied at recommended rates to trees during the season. Fruit with an approximate average diameter of 5–7 cm were picked at the maturity stage when fruit are normally harvested commercially and then used in the packing line tests the same day or held 1–4 days at 4 C until used.

At Byron and Clemson, conidia of

M. fructicola were added to the dump tank water to make approximately 10^3 spores/ml. The conidia were obtained from diseased fruit that had been inoculated or naturally infected with a benomyl-sensitive strain of *M. fructicola*. After conidia were added, the water was allowed to circulate for a minimum of 15 min before proceeding with the test. Because the packinghouse at Musella had a dry-dump system, inoculating fruit before the packing line treatment was not feasible. Therefore, a spore suspension (10^5 conidia/ml) was applied to the fruit with an airbrush 1 and 5 days after the packinghouse treatments.

The bacterial preparations were applied in the commercial fruit wax, Peach, Nectarine, and Plum Lustre 251 (Decco-Tiltbelt Division, Pennwalt Corp., Monrovia, CA), diluted one to three (v:v) with water (deionized) according to label instructions. Botran 75 WP (active ingredient, dicloran), used for *Rhizopus* control, was also combined with B-3 in the treatments. Benlate 50 WP (active ingredient, benomyl) was used as the standard brown rot fungicide. The active compound, dicloran, was applied at 2.1 or 5.25 mg/ml and benomyl was applied at 0.7 or 1.75 mg/ml. Although benomyl levels were in excess of the benomyl concentration for peaches (0.3 mg/ml) according to the Benlate label, the latter is generally inadequate to attain 1–2 ppm residue in fruit with packing line systems that apply fungicide in water base wax. Adherence to label instructions for Decco Salt. No. 20 (a mixture of dicloran and benomyl formulated for use in water base wax produced by Decco Tiltbelt) results in a concentration of 2.22 mg/ml of dicloran and 0.74 mg/ml of benomyl. Fruit treated on the packing lines had dicloran residues of 2.6–5.1 ppm and benomyl residues of 1.0–1.9 ppm.

Freshly harvested B-3 from flask cultures at Byron was applied at 5.0×10^8 cfu/ml and stored forms were applied at concentrations of 1×10^8 to 3×10^8 cfu/ml. Based on rates of dicloran applied in combination with B-3 and subsequent residue analyses, the estimated cfu added to each fruit was 7×10^7 and 2×10^7 cfu for fresh and stored B-3 from Byron, respectively. B-3 preparations from the fermentor culture at Brownsville were applied at 2×10^{10} cfu/ml. An estimated 2×10^9 cfu of this B-3 were added to each fruit.

At Clemson, replicates for one treatment were run over the packing line in succession, separated by tennis balls. At Byron and Musella, treated fruit were not separated into replicates until they were packed in boxes. Each replicate consisted of 100 fruit separated on 5 trays stacked in a box. Four replicates were used per treatment at Byron and Clemson, and six at Musella. The fruit were incubated at 23–25 C and evaluated on two separate dates. On the first date, those with brown rot and rhizopus rot were counted and removed, and the noninfected fruit were incubated for an additional 3–4 days. The total incidence of disease was calculated after the second observation. Data were analyzed by the analysis of variance and Duncan's multiple range test ($P = 0.05$).

Table 1. Application of *Bacillus subtilis* (B-3) from flask cultures on a simulated commercial packing line at Byron, GA, for peach brown rot control[v]

	Brown rot[x] (%)		Rhizopus rot[x] (%)	
Treatment[w]	**4 Days**	**8 Days**	**4 Days**	**8 Days**
Noninoculated check	1.0 c	17.6 c	2.1 d	7.5 cd
Water	44.5 a	69.3 a	52.4 a	66.7 a
Wax	15.8 b	59.2 ab	32.9 b	51.0 b
Dicloran[y] + wax	6.8 bc	43.8 b	9.9 c	15.8 c
Benomyl[y] + wax	0.0 c	1.8 c	6.8 cd	10.3 cd
Benomyl + dicloran + wax	0.3 c	8.6 c	6.6 cd	13.1 cd
B-3 fresh[z] + wax	0.3 c	3.5 c	5.8 cd	10.5 cd
B-3 fresh + dicloran + wax	0.5 c	2.0 c	6.5 cd	13.6 cd
B-3 stored[z] wet + dicloran + wax	1.0 c	9.1 c	6.8 cd	17.1 c
B-3 stored dry + dicloran + wax	0.5 c	10.0 c	1.5 d	4.9 d

[v] B-3 cultured in nutrient-yeast-dextrose broth in flasks and applied immediately (fresh) or stored at 4 C as paste (wet) or powder (dry).
[w] Fruit inoculated before treatment in dump tank with *Monilinia fructicola* at 10^3 conidia/ml.
[x] Percent of fruit with decay determined after 4 and 8 days at 23–25 C. Each value is a mean of four replicates of 100 fruit. Means with same letter in column are not different ($P = 0.05$), according to Duncan's multiple range test.
[y] Dicloran and benomyl applied at 2.1 and 0.7 mg/ml, resulting in 2.6 and 1.0 ppm residue in fruit, respectively.
[z] B-3 in fresh and stored forms applied at 5×10^8 and 1×10^8 cfu/ml to add approximately 7×10^7 and 2×10^7 cfu per fruit, respectively.

RESULTS

In the simulated packing line test at Byron, the incidence of brown rot was reduced as much with B-3 as with the

Table 2. Antibiotic production by *Bacillus subtilis* (B-3) in 14-L fermentors with media lower in cost and more suited for commercial fermentation than the nutrient-yeast-dextrose broth (NYDB) used in laboratory experiments

	Medium ingredients (g)[u]							Width of inhibition zone (mm) ± SE[x]		Diameter of lesion on fruit (mm)[y]
Medium	**Nutrient broth**	**Proflo[v]**	**Dextrose**	**Yeast extract**	**Peptone**	**Hy-Soy[w]**	**$CaCO_3$**	**36 hr**	**60 hr**	
None[z]	···	···	···	···	···	···	···	0.3 ± 0.30	0.3 ± 0.30	29.9 a
NYDB	8	···	10	5	···	···	···	4.6 ± 0.43	6.9 ± 0.19	9.4 ab
A	···	15	15	···	5	···	1	0	1.5 ± 0.42	···
B	···	30	15	5	5	···	1	0	0	21.6 ab
C	···	30	30	5	5	···	1	0	0.7 ± 0.70	···
D	···	15	30	5	5	···	1	0.4 ± 0.40	3.9 ± 0.19	7.2 b
E	···	···	20	5	···	20	1	0.3 ± 0.30	2.6 ± 0.24	16.2 ab

[u] All media also included: 0.3 g $MgSO_4$, 20 mg $FeSO_4 \cdot 7H_2O$, 20 mg $ZnSO_4 \cdot 7H_2O$, and 20 mg $MnSO_4 \cdot H_2O$.
[v] A partially defatted cooked cottonseed flour (Traders Protein Division of Traders Oil Mill Company, Fort Worth, TX).
[w] A defatted soybean meal flour (Sheffield Products, Memphis, TN).
[x] Antibiotic produced in 36-hr-old and 60-hr-old cultures was both extracted and tested against *M. fructicola* as described by McKeen et al (6). Crude extracts in 80% ethanol diffused from wells in agar medium and inhibited fungal spore germination in zone surrounding well. Values are means for five wells.
[y] Antibiotic extracts from 60-hr-old cultures were tested in March against *M. fructicola* on Chilean nectarines that were wounded. Ten fruit per treatment were incubated 6 days at 25 C. Means with same letter are not different ($P = 0.05$), according to Duncan's multiple range test.
[z] 80% ethanol was used as check in assays.

standard fungicide, benomyl (Table 1). Results for stored as compared with fresh preparations were similar. Brown rot appeared due largely to the inoculation. The noninoculated fruit had only 2 and 25% as much disease as the inoculated check fruit after 4 and 8 days, respectively. In addition, rhizopus rot incidence increased with inoculation made with *M. fructicola*. Moreover, when the incidence of brown rot was reduced by treatments, rhizopus rot also was reduced. Dicloran alone reduced rhizopus rot but not brown rot.

In preliminary tests conducted as a necessary step in the scale up of B-3 production, changes in the amounts or ratio of nitrogen and carbon sources were found to affect antibiotic production in culture (Table 2). Extracts from 60-hr-old cultures appeared to have greater antifungal activity than those from 36-hr-old cultures. The extract from the NYDB culture produced the widest zones of inhibition and the extract from the medium D culture ranked second. When tested in vivo against *M. fructicola*, the medium D and NYDB culture extracts were not different ($P = 0.05$).

The preparations of B-3 from cultures grown in medium D (Table 2) in the 250-L fermentor were comparable to that produced in flasks or to benomyl in the packing line test at Clemson (Table 3). However, brown rot incidence was generally low even though the inoculum level was comparable to that used at Byron. As in the Byron test, the incidence of brown rot from natural inoculum was low. Dicloran was again ineffective against brown rot when applied alone.

In the commercial packinghouse at Musella (Table 4) benomyl was superior to B-3 for control of brown rot. The B-3 preparation from flask cultures had about the same effect on brown rot as did benomyl over an 11-day incubation period, but was associated with an increased incidence of rhizopus rot. Neither the wet- nor the dry-stored B-3 from the culture grown in the 250-L fermentor reduced brown rot. Incidences of brown rot for these treatments, which included dicloran, were higher than the treatment with dicloran alone.

DISCUSSION

Because of the drought in 1986 when these tests were conducted, brown rot disease pressure was low. This was evident in all tests. Drought conditions also may have affected fruit susceptibility in the tests at Clemson and Musella in which brown rot incidence was low following single inoculations. Although positive results were obtained with *B. subtilis* for postharvest brown rot control on the simulated packing lines, tests will need to be conducted during more than 1 year to determine efficacy.

Results of the test in the packinghouse at Musella were not so favorable (Table 4). However, this test was probably less reliable than those on the simulated packing lines because of unusual circumstances. It was not feasible to completely remove fungicide residues from brushes and other equipment before and between treatments. This could have reduced the differences among treatments and it could be another explanation of why a second inoculation was necessary. The fact that inoculation was delayed may also have affected test results. If disease resulted primarily from the second inoculation and if the bacterium or its antibiotic was unstable on the fruit, brown rot control would break down. Possibly this occurred, particularly with the B-3 cultured at Brownsville. It was previously found (9) that B-3 produced in NYDB at Byron reduced brown rot even when fruit were treated and then stored at 2–4 C for 21 days before inoculation with *M. fructicola*. In addition, there may have been a difference in antibiotic production, either qualitative or quantitative. Although the Brownsville B-3 was in much greater cfu/ml concentration in culture, it may have produced less antibiotic in culture and on treated fruit. It appeared from assays in plates (Table 2), but not in vivo, that antibiotic production was lower after 36 and 60 hr in the medium selected for use at Brownsville compared with the NYDB medium used at Byron. The test at Musella was a first attempt to test in a commercial setting the postharvest application of a biological control agent to a food commodity for the purpose of controlling a disease.

The favorable effects with B-3, when freshly harvested from flask cultures and applied on the simulated packing line

Table 3. Application of *Bacillus subtilis* (B-3) from flask and fermentor cultures on a simulated packing line at Clemson, SC, for peach brown rot control[v]

Treatment[w]	Brown rot[x] (%)		Rhizopus rot[x] (%)	
	4 Days	7 Days	4 Days	7 Days
Noninoculated check	0 c	0.3 d	0.5 a	1.0 a
Water	2.5 bc	10.8 abc	0.3 a	0.5 a
Wax	3.3 ab	14.3 a	0.8 a	1.5 a
Dicloran[y] + wax	2.0 bc	13.3 ab	0 a	0 a
Benomyl[y] + dicloran + wax	0 c	2.5 cd	0 a	0 a
B-3 wet (BY)[z] + dicloran + wax	0 c	3.3 cd	0.3 a	0.8 a
B-3 wet (BR)[z] + dicloran + wax	0 c	4.0 bcd	0 a	0 a
B-3 dry (BR) + dicloran + wax	0 c	0.8 cd	0 a	0 a

[v] B-3 cultured in flasks at Byron, GA (BY), or in 250-L fermentor at Brownsville, TX (BR), and stored at 4 C as paste (wet) or powder (dry).
[w] Fruit inoculated before treatment in dump tank with *Monilinia fructicola* at 10^3 conidia/ml.
[x] Percent of fruit with decay determined after 4 and 7 days at 23–25 C. Each value is a mean of four replicates of 100 fruit. Means with same letter in column are not different ($P = 0.05$), according to Duncan's multiple range test.
[y] Dicloran and benomyl applied at 2.1 and 0.7 mg/ml, resulting in 3.75 and 1.4 ppm residue in fruit, respectively.
[z] B-3 from Byron and Brownsville applied at 3×10^8 and 2×10^{10} cfu/ml, to add approximately 2×10^7 and 2×10^9 cfu per fruit, respectively.

Table 4. Application of *Bacillus subtilis* (B-3) from flask and fermentor cultures in a commercial packinghouse in Musella, GA, for peach brown rot control[v]

Treatment[w]	Brown rot[x] (%)		Rhizopus rot[x] (%)	
	8 Days	11 Days	8 Days	11 Days
Water	11.9 b	45.3 b	1.0 a	4.7 ab
Wax	22.1 a	58.1 a	1.0 a	2.9 b
Dicloran[y] + wax	4.8 bc	27.8 c	0.3 a	1.9 b
Benomyl[y] + dicloran + wax	0.5 c	13.3 d	1.0 a	3.5 b
B-3 wet (BY)[z] + dicloran + wax	8.3 bc	20.6 cd	1.2 a	8.5 a
B-3 wet (BR)[z] + dicloran + wax	8.0 bc	46.6 ab	1.2 a	4.8 ab
B-3 dry (BR) + dicloran + wax	7.2 bc	52.4 ab	0.7 a	4.6 ab

[v] B-3 cultured in flasks at Byron, GA (BY), or in 250-L fermentor at Brownsville, TX (BR), and stored at 4 C as paste (wet) or powder (dry).
[w] Fruit spray-inoculated 1 and 5 days after treatment with 10^5 conidia/ml suspension of *Monilinia fructicola*.
[x] Percent of fruit with decay determined after 8 and 11 days at 23–25 C. Each value is a mean of six replicates of 100 fruit. Means with same letter in column are not different ($P = 0.05$), according to Duncan's multiple range test.
[y] Dicloran and benomyl applied at 5.25 and 1.75 mg/ml, resulting in 5.1 and 1.9 ppm residue in fruit, respectively.
[z] B-3 from Byron and Brownsville applied at 3×10^8 and 2×10^{10} cfu/ml, to add approximately 2×10^7 and 2×10^9 cfu per fruit, respectively.

(Table 1), were comparable to the effects observed with fresh material in previous experiments in the laboratory (8,9). Similar results with B-3 stored as a paste or powder before use in the packing line tests (Tables 1 and 3) demonstrated a necessary step for commercialization to become a reality.

Not only was the scale up from culture flasks to a 250-L fermentor desired in order to simulate commercial production of B-3, it facilitated the production of sufficient quantities of B-3 for the packing line tests. When stored B-3 produced at Byron was used in the tests, it consisted of a combination of culture batches harvested on more than one date. The only problem encountered when B-3 was grown in the 250-L fermentor was an unusually high degree of foaming. Several antifoam agents were tested before finding one that was effective (Dulmage, *unpublished*). Even then, to adequately deal with the problem, it was necessary to use a volume of medium below the normal operating capacity of the fermentor.

The prospect of commercial production and application of the B-3 strain of *B. subtilis* for postharvest control of peach brown rot was strengthened. Efficacy of this biological control agent against brown rot could be improved with further efforts to develop the fermentation and formulation technology.

LITERATURE CITED

1. Dulmage, H. T. 1971. Production of δ-endotoxin by eighteen isolates of *Bacillus thuringiensis*, serotype 3, in 3 fermentation media. J. Invertebr. Pathol. 18:353-358.
2. Gueldner, R. C., Reilly, C. C., Pusey, P. L., Costello, C. E., Arrendale, R. F., Cox, R. H., Himmelsbach, D. S., Crumley, F. G., and Cutler, H. G. 1988. Isolation and identification of Iturins as antifungal peptides in biological control of peach brown rot with *Bacillus subtilis*. J. Agric. Food Chem. 36:366-370.
3. Jones, A. L., and Ehret, G. R. 1976. Isolation and characterization of benomyl-tolerant strains of *Monilinia fructicola*. Plant Dis. Rep. 60:765-769.
4. Kilgore, W. W., Cheng, K. W., and Ogawa, J. M. 1962. Extraction and determination of 2,6-Dichloro-4-nitroaniline in processed fruits. J. Agric. Food Chem. 10:399-401.
5. Kirkland, J. J., Holt, R. F., and Pease, H. L. 1973. Determination of benomyl residues in soils and plant tissues by high-speed cation exchange liquid chromatography. J. Agric. Food Chem. 21:368-371.
6. McKeen, C. D., Reilly, C. C., and Pusey, P. L. 1986. Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicola* from *Bacillus subtilis*. Phytopathology 76:136-139.
7. Ogawa, J. M., Manji, B. T., Rough, D., and Sonoda, R. M. 1981. Monitoring and control of benomyl-resistant *Monilinia fructicola*. (Abstr.) Phytopathology 71:246.
8. Pusey, P. L., and Wilson, C. L. 1984. Postharvest biological control of stone fruit brown rot by *Bacillus subtilis*. Plant Dis. 68:753-756.
9. Pusey, P. L., Wilson, C. L., Hotchkiss, M. W., and Franklin, J. D. 1986. Compatibility of *Bacillus subtilis* for postharvest control of peach brown rot with commercial fruit waxes, dicloran, and cold-storage conditions. Plant Dis. 70:587-590.
10. Sleesman, J. P., and Leben, C. 1978. Preserving phytopathogenic bacteria at −70 C or with silica gel. Plant Dis. Rep. 62:910-913.
11. Szkolnik, M., and Gilpatrick, J. D. 1977. Tolerance of *Monilinia fructicola* to benomyl in western New York. Plant Dis. Rep. 61:651-657.
12. Wilson, C. L., and Pusey, P. L. 1985. Potential for biological control of postharvest plant diseases. Plant Dis. 69:375-378.
13. Zehr, E. I. 1982. Control of brown rot in peach orchards. Plant Dis. 66:1101-1105.