# Compatibility of *Bacillus subtilis* for Postharvest Control of Peach Brown Rot with Commercial Fruit Waxes, Dicloran, and Cold-Storage Conditions

P. L. PUSEY, Research Plant Pathologist, USDA-ARS, Southeastern Fruit and Tree Nut Research Laboratory, P.O. Box 87, Byron, GA 31008; C. L. WILSON, Research Plant Pathologist, USDA-ARS, Appalachian Fruit Research Station, Route 2, Box 45, Kearneysville, WV 25430; M. W. HOTCHKISS, Research Technologist, USDA-ARS, Southeastern Fruit and Tree Nut Research Laboratory, P.O. Box 87, Byron, GA 31008; and J. D. FRANKLIN, Research Technologist, USDA-ARS, Appalachian Fruit Research Station, Route 2, Box 45, Kearneysville, WV 25430

**ABSTRACT**

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*Bacillus subtilis* (strain B-3), previously shown effective against brown rot of harvested stone fruit, was tested further in the laboratory to determine whether the bacterium, as a substitute for a chemical fungicide, would be compatible with other postharvest agents or procedures used commercially. To wounded or nonwounded fruit, preparations of B-3 were applied in combination with commercial fruit waxes and dicloran, a fungicide used widely for *Rhizopus* control. Fruit were subsequently challenged with spores of *Monilinia fructicola* and incubated at 20 or 25 C. In a few instances, wax appeared to have a slight negative effect on the activity of B-3 against *M. fructicola*, but dicloran provided added protection against the fungus. When fruit treated with B-3 were subjected to simulated cold-storage conditions (2–4 C) for up to 21 days before the fungal-spore challenge and incubation at 20 C, antifungal activity was retained. The study indicates potential commercial application of *B. subtilis* for postharvest control of brown rot.

Additional key words: *Prunus persica*

The storage and marketing life of peaches (*Prunus persica* (L.) Batsch) is limited by postharvest diseases such as brown rot, caused by *Monilinia* spp. (2). Chemicals, particularly the benzimidazoles, have been used for preharvest and postharvest control of brown rot; however, these are less effective now because of the development of pathogen resistance (11).

Biological control may be an alternative to chemical control in the postharvest environment (9). Pusey and Wilson (7) successfully used aqueous cell suspensions of *Bacillus subtilis* (Ehrenberg) Cohn, strain B-3, to protect harvested peaches, plums, apricots, and nectarines from infection by *Monilinia fructicola* (Wint.) Honey. *B. subtilis* was also recently shown effective against decay fungi in postharvest treatments of citrus fruit (8).

In a commercial peach-packing operation, fruit are generally coated with waxes to improve fruit appearance, reduce moisture loss, and serve as a carrier for fungicides. The fungicides commonly incorporated into wax (either premixed by the wax producer or added by the packer) include benomyl (Benlate) for brown rot control and dicloran (Botran) for Rhizopus rot control. The packed fruit are transported as soon as possible and are generally held in cold storage for no longer than 2–3 wk before reaching the consumer. This study was conducted in the laboratory to determine whether the B-3 strain of *B. subtilis*, when substituted for benomyl or other chemicals used for postharvest brown rot control, would be compatible with commercial waxes and dicloran and whether it would retain its ability to control the disease under cold-storage conditions.

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## MATERIALS AND METHODS

All tests with fruit involved the B-3 strain of *B. subtilis* as the antagonist and an isolate of *M. fructicola* (WV-20) obtained from a peach orchard near Kearneysville, WV. Fruit from trees at the Southeastern Fruit and Tree Nut Research Laboratory, Byron, GA, and the Appalachian Fruit Research Station, Kearneysville, WV, included the cultivars Springcrest, Correll, Regina, Harvester, Loring, and Redskin. Peaches were picked at firm ripe maturity similar to those normally harvested commercially for storage or transit. Sulfur or mixtures of benomyl and sulfur or benomyl and captan were applied at recommended rates to trees during the season but no less than 4 days before harvest. The fruit were washed in water before the laboratory tests.

Because fungicide residues from field applications may affect the activity of B-3, fungicides commonly applied to trees in late season were tested for inhibitory action. A B-3 culture was grown in nutrient-yeast-dextrose broth (NYDB; 8 g of nutrient broth, 5 g of yeast extract, and 10 g of dextrose per liter of medium) on a rotary shaker at 250 rpm and 30 C for 48 hr, then 0.5 ml of suspension was spread with a bent-glass rod on nutrient-yeast-dextrose agar (NYDA; NYDB plus 15 g of agar per liter of medium). A sterilized paper disk (12.7 mm in diameter) was placed on the agar surface, and a 120-$\mu$l volume of the fungicide at concentrations of 0.25×, 0.5×, 1×, 2×, and 4× the recommended field rate was added to each of five replicate disks on separate plates. The fungicides tested and recommended rates for active ingredients were as follows: sulfur, 9.71 g $L^{-1}$; benomyl, 300 mg $L^{-1}$; thiophanate-methyl (Topsin M), 839 mg $L^{-1}$; captan (Orthocide 50), 2.27 g $L^{-1}$; and triforine (Funginex EC), 539 mg $L^{-1}$. After incubation at 25 C for 72 hr, inhibition of B-3 was evaluated by measuring the width of the clear zone (if present) between the disk and the bacterial growth.

**Fruit testing procedure.** Strain B-3 was stored on silica gel at −20 C (4). Before testing on fruit, the bacterium was grown on NYDA, then transferred to 50 ml of NYDB in 250-ml flasks. After incubation on a rotary shaker at 250 rpm and 30 C for 24 hr, 2 ml of this seed culture was transferred to 400 ml of NYDB in 2-L flasks. The final cultures were grown at 250 rpm and 30 C for 72 hr. A cell suspension was prepared by centrifuging the culture at 5,700 rcf for 20 min, resuspending the pelleted cells in deionized water, and centrifuging again to further remove extracellular solutes. The "rinsed" cells were suspended a second time in water, with the volume equal to that of the starting culture. Fruit were treated with the unaltered B-3 culture (cells not separated from growth medium) or the cell suspension in water, both consisting of $10^7$–$10^8$ colony-forming units per milliliter as determined by dilution plate counts.

Tests were performed with wounded or nonwounded fruit. One puncture wound per fruit (3 mm in diameter through the skin and 3 mm deep) was made as described previously (7). Treatments were applied with a paintbrush (2.5–4 cm wide), allowed to dry for 30–60 min, and then challenged with the *M. fructicola* spores collected from inoculated fruit. A volume of 20 $\mu$l of the fungal spore suspension ($10^5$ conidia per milliliter) was placed in the wound or on the fruit surface. On nonwounded fruit, the suspension drop formed a small bead that evaporated or was absorbed by the fruit within 1–2 hr. Ten fruits per treatment (each fruit considered as one replicate) were held on Styrofoam fruit trays (Soft-Pak tray, Amoco Foam Products, Yakima, WA) in plastic containers with lids (Le Beau Products, Baraboo, WI) that held up to 20 fruits. In each container, all treatments were represented in a randomized design. Fruit were incubated at 20 or 25 C (depending on whether the test was performed at Byron, GA, or Kearneysville, WV) and 70–100% relative humidity (RH). After periods of incubation extending from 3 to as many as 15 days in one test, decayed fruit were evaluated by measuring the distance between two margins of the decay lesion through the point of inoculation. Although this distance follows an arc rather than a straight line, we refer to it as the lesion diameter. For wounded fruit, figures were corrected by subtracting 3 mm, the diameter of the wound. When advancing margins of the lesion completely encircled the fruit, bringing about total decay, lesion diameters of 222 and 225 mm were used for wounded and nonwounded fruit, respectively, based on an estimated average of fruit circumference.

**Commercial waxes.** The bacterial preparations were tested on fruit in combination with four different waxes commonly applied to fruit in commercial packinghouses. Two of the waxes, both consisting of a mineral oil and paraffin base, were obtained from Durand-Wayland, Inc., LaGrange, GA (Special Peach, Plum, and Nectarine Wax Coating), and Freshmark Chemical Co., Orlando, FL (Fresh Wax 58P). Two water-based waxes were from Decco Tiltbelt Division, Pennwalt Corporation, Monrovia, CA (Peach, Plum, and Nectarine Lustr 251), and FMC Corp., Woodstock, VA (Stayfresh 707 Fruit Wax). Bacterial preparations were applied separately 1–2 hr before wax application or mixed with the wax (1:1, v/v) and applied at the same time. Combinations involving the aqueous bacterial preparations and oil-based wax were continually agitated to maintain a mixture during application to both wounded and nonwounded fruit.

**Wax and dicloran.** Wounded fruit were treated with mixtures of the bacterial preparations and dicloran (899 mg $L^{-1}$ final concentration), wax, or both dicloran and wax. Oil-based wax was mixed with one volume of water (as a check) or one volume of the aqueous bacterial preparation by constant agitation with a magnetic stirrer. The experiment was performed three times with oil-based wax (combined data were analyzed) and once with water-based wax. In another test, fruit received the same treatments using oil-based wax but were challenged with 20 $\mu$l of a suspension containing $10^5$ *Rhizopus* sp. spores per milliliter (obtained from decayed peach fruit at Byron, GA) rather than *M. fructicola*.

**Simulated cold storage.** In addition to the compatibility experiments, treated fruit were placed in a simulated cold-storage environment (2–4 C and 70–100% RH) to test retention of antifungal activity under such conditions. Wounded and nonwounded fruit were held in the low-temperature environment for up to 21 days before the fungal-spore challenge and high-temperature (20 C) incubation. A rifampicin-resistant isolate derived from B-3 by the procedure of Kloepper et al (3) was used in fruit treatments to facilitate positive identification of the organism after storage and incubation. The isolate was maintained on NYDA containing 50 $\mu$l/ml rifampicin.

## RESULTS

Only captan and triforine were inhibitory to B-3. Mean width of the inhibition zone ranged from 0.9 to 3.1 mm for captan at 0.57–9 g $L^{-1}$ and from 0.4 to 9.4 mm for triforine at 0.13–2.2 g $L^{-1}$. Preharvest sprays with captan probably had little or no effect on the postharvest fruit tests because captan has low systemic activity and surface residue was removed during washing.

Fruit infected naturally with *Rhizopus* spp. or fungi other than *Monilinia* were removed from the experiments. Although 15.5% of fruit were omitted in one test (first column in Table 1), in all other tests, only 2 and 0.5% were omitted because of *Rhizopus* and other decay fungi, respectively. An examination of lesion diameter data indicated certain trends. The only statistical comparisons for any one incubation period were based on the number of fruit decayed per total number of inoculated fruit not contaminated with other fungi, using the two-tailed Fisher's exact test ($P = 0.05$).

**Commercial waxes.** Although means for lesion diameters were generally lower for wax treatments than for the water check, a statistical separation was not shown based on number of fruit decayed (Table 1). Wax applied in combination with B-3 always resulted in a mean lesion diameter that was lower than the mean for the same wax applied alone; significant differences in numbers of decayed fruit were shown in all these treatments, except for a few involving the B-3 cell suspension combined with either Decco Tiltbelt or FMC wax. Mean diameters for combination treatments were often higher than those for B-3

**Table 1.** Compatibility of *Bacillus subtilis* (B-3) for peach brown rot control with commercial postharvest waxes[v]

| | Brown rot | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Wounded | | | | Nonwounded | | | |
| | Separate[x] | | Mixture[x] | | Separate | | Mixture | |
| Treatment[w] | D[y] | %[y] | D | % | D | % | D | % |
| Water | 71[z] | 100 abcd[z] | 85 | 100 a | 150 | 90 ab | 163 | 100 a |
| DW wax | 52 | 100 ab | 62 | 100 a | 87 | 90 ab | 43 | 100 a |
| FC wax | 49 | 90 abc | 50 | 100 a | 112 | 100 a | 46 | 100 a |
| DT wax | 62 | 100 ab | 69 | 100 a | 45 | 50 bc | 22 | 56 ab |
| FMC wax | 71 | 100 a | 81 | 100 a | 43 | 70 ab | 89 | 90 a |
| B-3 culture | 3 | 11 e | 1 | 10 d | 0 | 0 d | 0 | 0 b |
| B-3 culture + DW wax | 4 | 13 e | 2 | 11 d | 0 | 0 d | 0 | 0 b |
| B-3 culture + FC wax | 7 | 13 e | 3 | 10 d | 0 | 0 d | 1 | 10 b |
| B-3 culture + DT wax | 3[z] | 25 e[z] | 3 | 20 cd | 0 | 0 d | 0 | 0 b |
| B-3 culture + FMC wax | 5[z] | 33 de[z] | 15 | 40 bcd | 0 | 0 d | 1 | 0 b |
| B-3 cells | 12[z] | 43 cde[z] | 18 | 44 bcd | 0 | 0 d | 0 | 0 b |
| B-3 cells + DW wax | 13 | 40 cde | 11 | 44 bcd | 6 | 10 cd | 8 | 20 b |
| B-3 cells + FC wax | 6 | 30 de | 35 | 70 abc | 0 | 0 d | 1 | 20 b |
| B-3 cells + DT wax | 17 | 44 cde | 42 | 90 ab | 1 | 11 cd | 0 | 0 b |
| B-3 cells + FMC wax | 20 | 50 abcde | 47 | 89 ab | 0 | 0 d | 1 | 10 b |

[v] After treatment and inoculation, fruit were incubated in moist chamber at 20 C, wounded fruit for 6 days and nonwounded fruit for 9 days.

[w] B-3 culture (cells not separated from growth medium) and B-3 cells suspended in water were tested on fruit alone and in combination with oil-based waxes produced by Durand-Wayland, Inc. (DW) and Freshmark Chemical Co. (FC) and water-based waxes produced by Decco Tiltbelt Division of Pennwalt Corp. (DT) and FMC Corp. (FMC).

[x] B-3 and wax were applied at separate times (the former 1–2 hr before the latter) or as a mixture.

[y] D = mean diameter of decay lesion (mm); % = percentage of total fruit decayed. Statistical separation is based on numbers of decayed fruit per total fruit, using two-tailed Fisher's exact test; same letter in column indicates no difference ($P = 0.05$).

[z] Value is mean or percentage of five to seven fruits; all other values in table are for eight to 10.

applied alone, but no differences were found based on number of fruit decayed.

**Wax and dicloran.** Dicloran, when mixed with B-3 or B-3 plus wax, contributed to a reduction in brown rot. In the test with oil-based wax, dicloran alone or the B-3 cell suspension alone did not significantly reduce the number of fruit decayed, but a combination of the two did (Fig. 1). *Rhizopus* did not appear to be affected by B-3, but dicloran or combinations that included dicloran reduced the number of fruit decayed by this fungus.

When water-based wax was substituted for oil-based wax and fruit were observed over a 15-day incubation period, all check fruit treated with water or wax were totally decayed by *M. fructicola* after 9 days (Table 2). In contrast, only a few fruit treated with the culture, the culture plus dicloran, or the culture plus wax plus dicloran showed decay even after 15 days. Wax was shown after 9 days to adversely affect the activity of the B-3 culture, but the addition of dicloran compensated for this effect. The greater effectiveness of the culture alone compared with the cell suspension alone was evident after 12 and 15 days of incubation. On the basis of analysis of number of decayed fruit for each incubation period, combination treatments that included the cell suspension were equal in effectiveness to the cell suspension alone. However, when lesion diameter data collected over time were subjected to linear regression analysis, the slope for the mixture with cell suspension and dicloran was significantly lower ($P = 0.01$) than the slope for either the cell suspension alone or dicloran alone. The same differences were shown when these materials were applied in wax.

**Simulated cold storage.** When fruit treated with B-3 were held in simulated cold storage before fungal-spore challenge and high-temperature incubation, all treatments to nonwounded fruit prevented decay after 7 and 14 days of storage and reduced the number of decayed fruit after 21 days of storage (Table 3). All treatments to wounded fruit reduced the number of decayed fruit after 7 days of storage, but this was not shown for either of the B-3 preparations after 14 days of storage or the B-3 cell suspension after 21 days of storage. Benomyl always reduced the number of decayed fruit, except when wounded fruit were stored for 21 days. The rifampicin-resistant B-3 strain could still be isolated from fruit after 21 days of storage. This was confirmed in activity tests on fruit.

## DISCUSSION

In general, the B-3 strain of *B. subtilis* was compatible with commercial fruit waxes, dicloran, and simulated cold-storage conditions. There appeared to be a negative effect of wax on B-3 activity in some instances. This may reflect a reduced mobility of inhibitory compounds produced by the bacterium. Dicloran, on the other hand, actually provided added protection against *Monilinia* infection.

Other findings not reported previously (7) include positive results with nonwounded fruit as well as wounded fruit (Tables 1 and 3). Treatment of wounds with B-3 has been the preferred method of testing because we consider it more stringent. As expected in this study, results were more favorable with nonwounded fruit.

It is thought that the greater effect of the culture, compared with the cell suspension, is due to the action of metabolites present in the medium. A heat-stable substance extracted from B-3

**Table 2.** Compatibility of *Bacillus subtilis* (B-3) for postharvest brown rot control with dicloran and water-based wax[w]

| | Brown rot (days of incubation) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 3 | | 6 | | 9 | | 12[y] | | 15[y] | |
| Treatment[x] | D[z] | %[z] | D | % | D | % | D | % | D | % |
| Water | 59 | 100 a | 181 | 100 a | 222 | 100 a | 222 | 100 a | 222 | 100 a |
| Wax | 47 | 90 ab | 135 | 100 a | 222 | 100 a | 222 | 100 a | 222 | 100 a |
| Dicloran | 2 | 40 bc | 10 | 100 a | 20 | 100 a | 60 | 100 a | 105 | 100 a |
| Wax + dicloran | 13 | 100 a | 18 | 100 a | 33 | 100 a | 71 | 100 a | 117 | 100 a |
| B-3 culture | 0 | 0 c | 0 | 10 bc | 1 | 10 cd | 2 | 10 c | 3 | 20 b |
| B-3 culture + wax | 3 | 22 c | 21 | 56 ab | 74 | 78 a | 128 | 78 ab | 167 | 89 a |
| B-3 culture + dicloran | 0 | 0 c | 0 | 0 c | 0 | 0 d | 1 | 13 c | 2 | 14 b |
| B-3 culture + wax + dicloran | 1 | 10 c | 1 | 10 bc | 3 | 11 cd | 8 | 22 bc | 9 | 33 b |
| B-3 cells | 5 | 10 c | 5 | 22 bc | 26 | 63 abc | 146 | 86 ab | 171 | 100 a |
| B-3 cells + wax | 6 | 30 c | 25 | 60 ab | 95 | 80 a | 184 | 100 a | 200 | 100 a |
| B-3 cells + dicloran | 0 | 0 c | 1 | 10 bc | 2 | 20 bcd | 10 | 30 bc | 39 | 60 ab |
| B-3 cells + wax + dicloran | 1 | 10 c | 1 | 10 bc | 10 | 70 ab | 32 | 90 a | 41 | 90 a |

[w] After treatment and inoculation, wounded peach fruit were incubated in moist chamber at 20 C for 3–15 days.
[x] B-3 culture (cells not separated from growth medium) and B-3 cells suspended in water were tested on fruit alone and in combinations involving dicloran and water-based wax produced by Decco Tiltbelt Division of Pennwalt Corp.
[y] Values represent mean or percentage of seven to 10 fruits; in other columns, values represent eight to 10 fruits.
[z] D = mean diameter of decay lesion (mm); % = percentage of total fruit decayed. Statistical separation is based on numbers of decayed fruit per total fruit, using two-tailed Fisher's exact test; same letter in column indicates no difference ($P = 0.05$).

**Table 3.** Retention of *Bacillus subtilis* (B-3) activity against brown rot under simulated cold-storage conditions[w]

| | Brown rot (days of cold storage) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | | 7 | | | | 14 | | | | 21 | | | |
| | W[y] | | N | | W | | N | | W | | N | | W | | N | |
| Treatment[x] | D[z] | %[z] | D | % | D | % | D | % | D | % | D | % | D | % | D | % |
| Water | 96 | 100 a | 70 | 100 a | 83 | 100 a | 44 | 100 a | 98 | 90 a | 68 | 100 a | 172 | 100 a | 121 | 100 a |
| B-3 culture | 0 | 0 b | 0 | 0 b | 0 | 0 c | 0 | 0 b | 14 | 80 a | 0 | 0 b | 2 | 33 b | 6 | 10 b |
| B-3 cells | 7 | 10 b | 0 | 0 b | 15 | 50 b | 0 | 0 b | 20 | 70 a | 0 | 0 b | 51 | 100 a | 3 | 20 b |
| Benomyl | 4 | 20 b | 1 | 10 b | 3 | 10 bc | 0 | 0 b | 3 | 10 b | 0 | 0 b | 2 | 30 a | 1 | 0 b |

[w] After treatment, wounded (W) and nonwounded (N) fruit were held at 2–4 C for periods of 0–21 days. All fruit were then challenged with fungal spores and incubated at 20 C for 6 days.
[x] Fruit treated with B-3 culture (cells not separated from growth medium), B-3 cells suspended in water, or benomyl at 300 mg $L^{-1}$
[y] Values represent mean or percentage of eight to 10 fruits; in other columns, values represent 10 fruits.
[z] D = mean diameter of decay lesion (mm); % = percentage of total fruit decayed. Statistical separation is based on numbers of decayed fruit per total fruit, using two-tailed Fisher's exact test; same letter in column indicates no difference ($P = 0.05$).

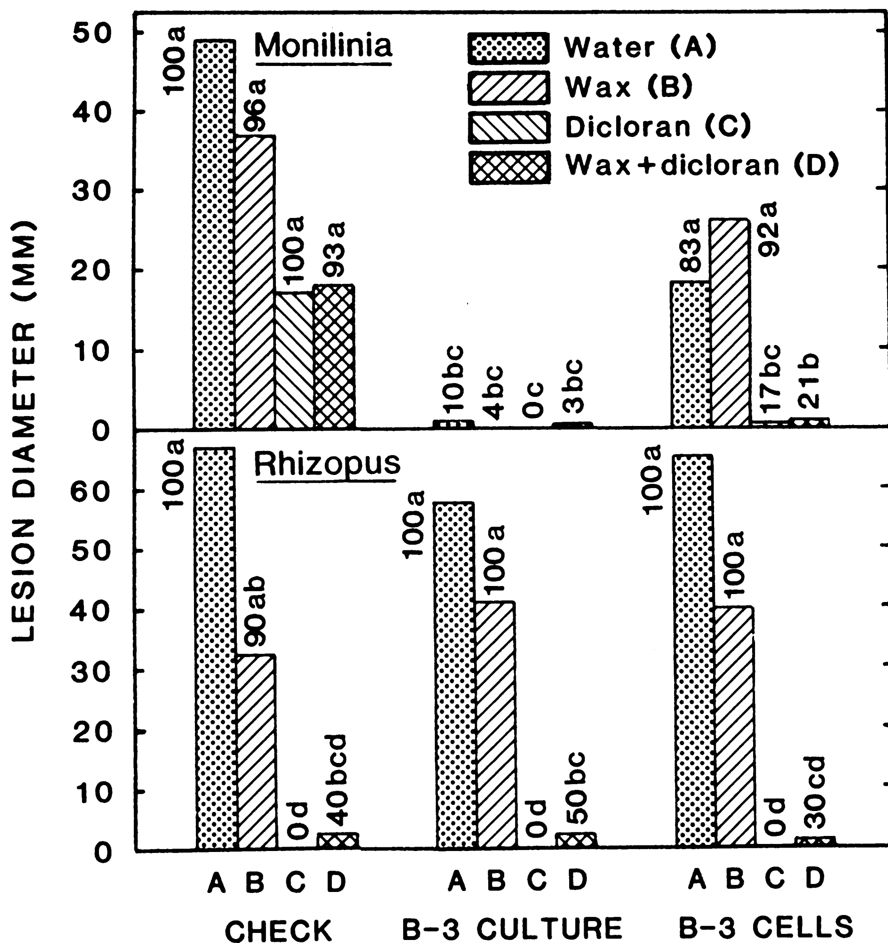


**Fig. 1.** Compatibility of *Bacillus subtilis* (B-3) as an agent in postharvest brown rot control with commercial oil-based wax (produced by Durand-Wayland, Inc.) and dicloran. The B-3 culture (cells not separated from growth medium) and B-3 cells suspended in water were tested on wounded peach fruit alone and in combination with water, wax, dicloran, or wax plus dicloran. Fruit were challenged with *Monilinia fructicola* or *Rhizopus* sp. and incubated for 3 and 2 days, respectively, in moist chamber at 25 C. For *Monilinia*, each bar represents mean lesion diameter (mm) for 25–30 fruits and for *Rhizopus*, each bar represents mean for 10 fruits. Value above bar is the percentage of total fruit decayed. Although percentage is expressed, statistical separation is based on numbers of decayed fruit per total fruit, using two-tailed Fisher's exact test; same letter indicates no difference ($P = 0.05$).

cultures was inhibitory to *M. fructicola* on fruit (5; R. C. Gueldner, C. C. Reilly, and P. L. Pusey, *unpublished*). At least six active compounds can be isolated, and their partial characterization suggests similarity to the iturin antibiotics isolated from *B. subtilis* cultures by French workers (1,6,10). The iturins have a wide antifungal spectrum, and in clinical trials on man and animals, they were shown to have a low toxicity and low allergenic effect (1).

It might be questioned whether the B-3 cells produced the inhibitor after being applied to fruit. Even though cells were "rinsed" to remove extracellular solutes, some inhibitor could be tied up in the cell and released at time of death. Previous work (7) indicated, however, that autoclaving the cell suspension completely destroyed activity but that autoclaving the culture filtrate only reduced activity. Although the bacterium was detected on fruit after 21 days, we do not know whether the organism was actively dividing to form new cells or merely persisted in the form of dormant spores.

It appears that the B-3 strain of *B. subtilis* or its antibiotic could potentially be incorporated for brown rot control in a commercial packing operation by the same methods currently used to apply fungicides. The organism or its product might be applied before waxing, during waxing, or without waxing and still provide protection against *M. fructicola*. Of course, antibacterial agents, which are sometimes included in wax formulations (e.g., orthophenylphenol), should be avoided if *B. subtilis* is used for biological control. The bacterial antagonist might also be used in the hydrocooling water just as fungicides are sometimes applied at this stage if fruit are stored in bulk temporarily before packing; however, use of the bacterium would not be compatible with the simultaneous use of hypochlorous acid, which is frequently included to reduce microbial activity.

Although the laboratory study demonstrated *B. subtilis* activity against brown rot in the presence of wax and dicloran, further testing to establish efficacy must be done in an actual or simulated commercial packinghouse.

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