Evaluation of Bacterial Epiphytes Isolated from Avocado Leaf and Fruit Surfaces for Biocontrol of Avocado Postharvest Diseases

L. Korsten, E. S. De Jager, and **E. E. De Villiers,** Department of Microbiology and Plant Pathology, **A. Lourens,** Department of Statistics, and **J. M. Kotzé** and **F. C. Wehner,** Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria 0002, Republic of South Africa

ABSTRACT

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Bacteria isolated from Fuerte avocado leaf and fruit surfaces were evaluated for in vitro antagonism toward *Dothiorella aromatica.* Thirty-three bacteria exhibiting pronounced growth inhibition were further tested for antibiosis against *Colletotrichum gloeosporioides, Thyronectria pseudotrichia, Phomopsis perseae, Pestalotiopsis versicolor,* and *Fusarium solani.* Optimum disease-reducing concentrations of *Bacillus subtilis* (isolate B246) exhibiting the highest degree of antibiosis were determined according to a checkerboard-type titration assay, by artificial inoculations on Fuerte and Edranol avocado fruit in the laboratory. Various concentrations (10^5, 10^6, 10^7, and 10^8 cells ml^{-1}) of *B. subtilis* were also incorporated into commercial Tag-wax and applied to Hass avocado fruit in the packinghouse for control of anthracnose, *Dothiorella/Colletotrichum* fruit rot complex (DCC), and stem-end rot (SE). In the artificial inoculation study, increasing concentrations of *B. subtilis* were effective against increasing concentrations of *C. gloeosporioides, F. solani,* and *T. pseudotrichia.* Control of *D. aromatica* was significant at the lower (10^3 and 10^4 cells ml^{-1}) pathogen concentrations, whereas inhibition of *P. perseae* and *P. versicolor* was more readily achieved at the lower (10^5 and 10^6 cells ml^{-1}) antagonist concentrations. In the packinghouse, a *B. subtilis* concentration of 10^7 cells ml^{-1} significantly reduced anthracnose and SE externally and internally, while the lower *B. subtilis* concentrations (10^5 and 10^6 cells ml^{-1}) were effective against internal DCC. Based on treatment means of all external and internal postharvest disease data, all *B. subtilis* concentrations performed equally well in controlling postharvest diseases.

Additional keywords: phylloplane, fructoplane

In South Africa, avocado (*Persea americana* Mill.) is susceptible to both preharvest (black spot, sooty blotch, and sooty mold) and postharvest (anthracnose, stem-end rot [SE], and *Dothiorella/Colletotrichum* fruit rot complex [DCC]) fruit diseases. Postharvest diseases are mainly caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz., which is associated with anthracnose, DCC, and SE; *Dothiorella aromatica* (Sacc.) Petrak & Sydow, which causes DCC and SE; and *Thyronectria pseudotrichia* (Schw.) Seeler, *Phomopsis perseae* Zerova, *Fusarium solani* (Mart.) Appel & Wr. emend. W.C. Snyder & H.N. Hans., *Pestalotiopsis versicolor* (Speg.) Steyart, *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., *Fusarium decemcellulare* C. Brick, *Fusarium sambucinum* Fuckel, *Drechslera setariae* (Sawada) Subram. & Jain, and *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill., all implicated in SE (8). The latter five organisms are considered to be of minor importance (8).

Since the majority of fruit is exported by sea, which inevitably entails long periods in storage, postharvest losses are of great concern to the avocado industry. Limited control can be achieved by preharvest spraying with fungicides such as benomyl and copper oxychloride, or with postharvest prochloraz applications. However, since the latter compound is not registered for use on fruit exported to France (19), the use of effective orchard management practices aimed at lowering the inoculum is the only alternative. Furthermore, buildup of pathogen resistance with the continuous use of benomyl (7) and visible copper residues on harvested fruit that have to be removed manually in the packinghouse, as well as growing awareness of the negative effects of chemicals on the environment and human health, have necessitated a search for alternative nonchemical methods such as biological control (13).

Biocontrol of fruit and leaf diseases through the use of antagonistic microorganisms has recently emerged as a viable disease management strategy (2,13,15,17,-32,33,38). Selection and screening of antagonistic microorganisms was extensively discussed by Andrews (1), Baker and Cook (3), and Cook and Baker (5).

With the exception of preliminary reports by the first author (18–21), there are no publications on biocontrol of avocado fruit diseases. The purpose of this study was to isolate bacteria associated with avocado leaf and fruit surfaces, to evaluate their inhibitory action in vitro against various postharvest pathogens, and to evaluate the most inhibitory isolates in the laboratory and packinghouse for control of avocado postharvest diseases.

MATERIALS AND METHODS

Isolation of bacterial epiphytes. Leaves were collected during April and October 1986 from five randomly selected Fuerte trees at Westfalia Estate, northern Transvaal province. With each sampling, 10 undamaged leaves of approximately the same size were picked at four points around each tree representing north, east, south, and west. The leaves, handled only by the petiole, were placed in paper bags and taken to the laboratory for immediate processing. In addition, 20 mature fruit picked randomly from the same trees midway through the harvesting season (May) were placed in packing boxes for processing.

Twenty leaf disks (two per leaf) were cut with a no. 10 corkborer from each set of 10 leaves. Disks were placed in McCartney bottles, each containing 9 ml of sterile quarter-strength Ringer's solution (commercial isotonic diluent, Merck [Pty] Ltd., Midrand, SA) supplemented with 0.1% Tween 80. To dislodge bacteria, the capped bottles were partially submerged in an ultrasonic water bath (UMC 5 Ultrasonic Manufacturing Company, Krugersdorp, SA) and sonicated for 15 s (optimized isolation procedure, unpublished data). A dilution series was made of each sample solution, and 0.1 ml each of the 10^4, 10^5, and 10^6 dilutions was spread onto Standard I (STD) agar (Biolab, Merck) containing 0.01% cycloheximide (Actidione, Merck) to inhibit fungal growth.

Fruit was placed individually in 1-liter glass beakers containing 500 ml of Ringer's. Beakers were covered with parafilm and partially submerged in an ultrasonic water bath for 20 s (optimized isolation procedure, unpublished data).

Corresponding author: L. Korsten
E-mail: LKORSTEN@agric.up.ac.za

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The sonified fruit washing of four fruit was combined and concentrated to a retention volume of 10 ml in a Minitan ultrafiltration unit (Millipore, Johannesburg, SA) with a 0.22-µm filter. Subsequent dilution and plating were done as before.

Plates were incubated at 24°C for up to 72 h, after which colonies were grouped according to macroscopic appearance, and representatives of each group were isolated. All isolated bacteria were maintained on STD slants and kept at 5°C.

In vitro screening of bacterial epiphytes for antagonism. Avocado postharvest pathogens *C. gloeosporioides*, *D. aromatica*, *T. pseudotrichia*, *P. perseae*, *Pestalotiopsis versicolor*, and *F. solani* (8) were freshly isolated, identified, and their pathogenicity confirmed before being used in the in vitro antagonism test. All bacteria isolated were initially screened in vitro for antagonism against one of the most important avocado postharvest pathogens, *D. aromatica*, by the dual culture technique (10). Disks of *D. aromatica* cultivated on potato-dextrose agar (PDA) (Biolab) were placed off-center on nutrient agar (NA) (Biolab) plates. After 3 days growth at 25°C, plates were streak-inoculated 45 mm from the fungal disk with the particular bacterial isolate. Plates not inoculated with bacteria served as controls. Percent growth inhibition was determined after 21 days by the formula of Skidmore (34): $K_r - r_1/k_r \times 100 = GI$, where K_r represents the distance (measured in mm) of fungal growth from the point of inoculation to the colony margin on control plates, r_1 the distance of fungal growth from the point of inoculation to the colony margin in the direction of the antagonist, and *GI* the percent growth inhibition. Percent growth inhibition was categorized on a scale from 0 to 4, where 0 = no growth inhibition, 1 = 1 to 25% growth inhibition, 2 = 26 to 50% growth inhibition, 3 = 51 to 75% growth inhibition, and 4 = 76 to 100% growth inhibition. After screening, 33 strains that inhibited growth of *D. aromatica* more than 25% were evaluated further against *C. gloeosporioides*, *D. aromatica*, *T. pseudotrichia*, *P. persea*, *Pestalotiopsis versicolor*, and *F. solani*.

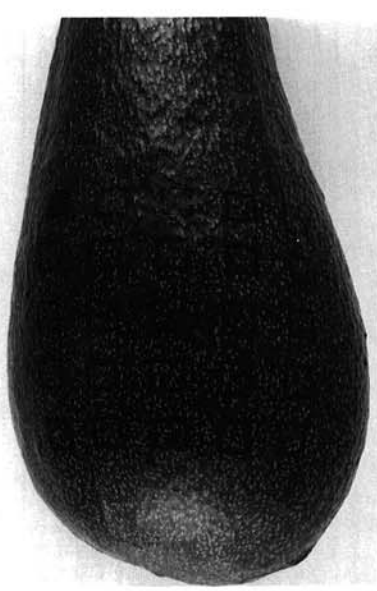

Fig. 1. Checkerboard pattern drawn on avocado fruit to determine the effect of antagonist versus pathogen concentration (checkerboard-type titration assay).

The 33 bacteria exhibiting antibiosis were identified with the API System (API International) using the API 20E (for *Enterobacteriaceae* and other gram-negative rods), 20NE (for non-*Enterobacteriaceae*), and 50CHB (for *Bacillus* spp.), as well as with key identification tests based on Krieg and Holt (23), Schaad (31), Sneath et al. (35), and Trüper and Krämer (36).

Evaluation and optimization of *Bacillus subtilis* in the laboratory. *Bacillus subtilis* (Ehrenberg) Cohn (B246), previously shown to inhibit growth of the major avocado postharvest pathogens in vitro, was cultured in 500-ml Erlenmeyer flasks containing 250 ml of STD broth. After 48-h shake-incubation (rotary shaker, 67 rpm) at 28°C, cells were harvested by centrifugation for 20 min at 16,080 × *g*. The resulting pellet was dissolved in 250 ml of Ringer's, cell concentration was determined with a Petroff-Hausser counting chamber, and a dilution series was made to concentrations of 10^8, 10^7, 10^6, and 10^5 cells ml^{-1}.

Avocado pathogens *C. gloeosporioides*, *D. aromatica*, *T. pseudotrichia*, *P. perseae*, *Pestalotiopsis versicolor*, and *F. solani* were cultured on PDA for 14 days at 25°C under a near-UV light source. Spores were harvested in Ringer's, counted with a hemacytometer, and diluted to obtain a concentration range from 10^3 to 10^6 spores ml^{-1} in 10-fold increments.

Early season mature Fuerte avocado fruit were used to determine the most effective antagonist concentration. The following procedure was used: squares (5 × 5 mm) were drawn with a black waterproof pen on one side of each fruit to give five vertical and five horizontal rows forming a checkerboard pattern of 25 squares (Fig. 1). The skin in each square was prick-wounded centrally to a depth of 5 mm with a sterile 1-mm-diameter inoculation needle, after which 20 µl of the various *B. subtilis* concentrations were applied by micropipette to each square. Starting from the left, squares in the vertical rows received suspensions of 10^8, 10^7, 10^6, and 10^5 cells ml^{-1}, respectively. The last row served as a control and received Ringer's. Fruit was left to air-dry at room temperature before being challenged with the various pathogen concentrations. Squares in the top horizontal row were inoculated with 20 µl of the pathogen suspension at 10^6 conidia ml^{-1}, in the following rows with 10-fold declining concentrations, and the last row received Ringer's only. Three fruit were used for each antagonist–pathogen combination. Inoculated fruit were randomly packed in cardboard boxes lined with absorbent paper and moistened cotton wool to maintain humidity. Boxes were stored at 25°C, and disease development was determined after 7 days.

Lesion surface area and disease severity rating were used as evaluation criteria. Lesion surface area was determined by measuring the diameter of the lesion in two different directions, obtaining the mean, and calculating the surface area (πr^2). Disease severity rating was done on an arbitrary 0 to 5 scale, where 0 = no disease development, 1 = browning of the lesion periphery and onset of necrosis, 2 = necrotic expansion spreading from the wound, 3 = necrosis of half the square, 4 = necrosis of the whole square, and 5 = necrotic expansion beyond the square. For confirming the efficacy of antagonists, the entire experiment was repeated on Fuerte and Edranol avocado fruit, also with three replicates.

Analysis of variance was performed on arcsine transformed lesion surface area data from single fruit replicates with the General Linear Model (GLM) procedure of Statistical Analysis System (SAS) (30). Regression analysis was used for each pathogen to obtain a predictive model of dose response. Spearman's correlation coefficients were used to correlate the two types of evaluation criteria, lesion surface area and disease severity ratings, and also to correlate the Fuerte and Edranol artificial infection experiments. Arcsine transformed data from the artificial infection experiments on Fuerte were combined for all six pathogens at each inoculum dosage, and the average lesion surface area value was plotted by computer program to simulate the effect of the various antagonist concentrations on the combined pathogens used in this study. All statistical analyses with probability values equal to or less than 0.05 were regarded as significant.

Evaluation and optimization of *B. subtilis* in the packinghouse. Mass cell production was achieved by inoculating fifty 250-ml Erlenmeyer flasks each containing 200 ml of STD broth with *B. subtilis* (B246). After 24 h of shake-incubation (67 rpm) at 28°C, each starter culture was added in its entirety to a 2-liter Erlenmeyer flask containing 1 liter of STD broth. Flasks were incubated (c. 48 h) as before until cell density reached 10^8 cells ml^{-1}, whereupon the contents were concentrated by the use of a Pellican Cassette filtration system (Millipore) with 100 000 MW filter. The resultant suspension was further concentrated by centrifugation as described previously. Pellets were pooled and frozen overnight before being lyophilized, sealed under vacuum in plastic bags, and transported to Westfalia Estate for immediate use. Lyophilized antagonist

powders were mixed into Tag-wax (polyethylene) (ICI [SA] [Pty] Ltd., Woodmead, SA) to obtain final concentrations of 10^5, 10^6, 10^7, and 10^8 cells ml^{-1}. For each treatment, three picking crates containing between 130 and 140 freshly picked late-season Hass fruit were randomly selected from the off-loading ramp in the packinghouse. At this stage, Hass was the only cultivar still in production. The three crates for each experiment were tilted onto the packing line, and the fruit were sprayed with one of the antagonist Tag-wax concentrations (1 liter per tonne of fruit) while rotating on nylon roller brushes. Rollers were changed and washed between treatments. Control fruit was Tag-waxed as for commercial purposes. Fruit was air-dried in the drying tunnel at 50°C for 2 min, packed in commercial boxes, and stored at 5.5°C for 28 days at Westfalia Estate before being transported to the laboratory for ripening and evaluation. Ripening took place at ambient temperature (22 to 28°C) for 7 days, and postharvest disease severity was evaluated at ready-to-eat ripeness. Each fruit was assessed for external anthracnose and DCC, and for internal anthracnose, DCC, and SE. Disease severity was rated on a 0 to 10 scale, with 0 being healthy and 10 indicating entire fruit decay (8). Data were statistically analyzed with SAS (30), by analysis of variance, and the significance of the treatment differences was determined using Duncan's multiple range test.

RESULTS

The identity and in vitro inhibitory effect of 33 bacteria against avocado postharvest pathogens are presented in Table 1. *Bacillus megaterium* (B91), *Bacillus* sp. (B92), *Bacillus cereus* (B247), *B. cereus* (B249), *B. subtilis* (B246), and *Bacillus licheniformis* (B248) were the only bacterial strains capable of inhibiting the growth of all six postharvest avocado pathogens. Of these, *B. subtilis* (B246) was the most inhibitory toward all pathogens tested. In vitro growth of *T. pseudotrichia* was reduced by eight bacterial strains, *Pestalotiopsis versicolor* by 11, and *F. solani* by 16. *D. aromatica* was inhibited to a lesser or greater extent by all 33 bacteria evaluated, having the highest mean GI category score of 1.7.

The effect of varying pathogen and antagonist concentrations on biocontrol efficacy using single fruit replicates is shown in Figures 2 to 7. Increasing concentrations of *B. subtilis* significantly reduced disease (lesion surface area) resulting from infection with increasing concentrations of *C. gloeosporioides* ($P = 0.0001$), *F. solani* ($P = 0.0004$), and *T. pseudotrichia* ($P = 0.0174$). Control of *D. aromatica* was effective at the two lowest pathogen concentrations (10^3, 10^4) ($P = 0.0001$ and $P = 0.0013$), while control of *P. perseae* ($P = 0.0001$ and $P = 0.0058$) and *Pestalotiopsis versicolor* ($P = 0.0001$ and $P = 0.001$) was

Table 1. Identity and effect of bacteria isolated from avocado leaf and fruit surfaces on in vitro growth of six postharvest avocado pathogens

		Growth inhibition (GI) category[z]							
Bacterial taxon	**Isolate**	***Colletotrichum gloeosporioides***	***Dothiorella aromatica***	***Thyronectria pseudotrichia***	***Phomopsis perseae***	***Pestalotiopsis versicolor***	***Fusarium solani***	**Mean GI category**	**Pathogens inhibited**
Agrobacterium radiobacter	B103	0	1	0	1	0	0	0.3	2
Bacillus sp.	B80	1	2	0	1	0	0	0.7	3
Bacillus sp.	B92	1	2	1	1	1	1	1.2	6
Bacillus sp.	B106	1	1	0	1	1	1	0.8	5
Bacillus cereus	B79	2	3	0	1	1	1	1.2	5
B. cereus	B87	1	1	0	2	0	1	0.8	4
B. cereus	B102	1	2	0	1	1	1	1.0	5
B. cereus	B108	2	1	0	0	1	1	0.8	4
B. cereus	B247	3	3	1	1	2	2	2.0	6
B. cereus	B249	2	2	1	1	1	1	1.3	6
Bacillus licheniformis	B248	2	3	1	1	1	2	1.8	6
Bacillus megaterium	B88	1	2	0	1	1	1	1.0	5
B. megaterium	B91	1	2	1	1	1	1	1.2	6
B. megaterium	B105	0	1	0	1	0	1	0.5	3
Bacillus mycoides	B94	1	2	0	1	1	1	1.0	5
Bacillus pumilus	B100	1	2	0	1	0	1	0.8	4
B. pumilus	B109	1	2	0	1	0	0	0.7	3
Bacillus subtilis	B246	2	2	2	2	1	3	2.3	6
Corynebacterium sp.	B95	0	1	0	0	0	0	0.2	1
Corynebacterium sp.	B96	0	2	0	0	0	0	0.3	1
Erwinia sp.	B82	0	1	0	0	0	0	0.2	1
Pseudomonas sp.	B81	0	2	0	2	0	0	0.7	2
Pseudomonas fluorescens	B110	1	1	0	1	0	0	0.7	4
Pseudomonas paucimobilis	B84	1	2	0	1	0	0	0.7	3
P. paucimobilis	B89	0	1	0	0	0	0	0.2	1
Pseudomonas putida	B85	1	1	0	1	0	0	0.5	3
P. putida	B93	2	2	1	0	0	0	0.8	3
P. putida	B97	1	2	0	1	0	0	0.7	3
Pseudomonas vesicularis	B104	0	1	0	0	0	0	0.2	1
Staphylococcus xylosus	B107	0	1	0	0	0	0	0.2	1
Vibrio fluvialis	B112	1	2	0	0	0	0	0.5	2
Xanthomonas maltophilia	B90	0	2	1	1	1	0	0.7	4
X. maltophilia	B99	0	2	0	1	0	0	0.5	2
Mean GI category score		0.9	1.7	0.3	0.8	0.4	0.6		
Total inhibitory bacteria		22	33	8	24	13	16		

[z] Percent growth inhibition was determined after 21 days by the formula of Skidmore (34). Values were categorized on a scale from 0 to 4, where 0 = no growth inhibition, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75%, and 4 = 76 to 100%.

more readily achieved with the lower antagonist concentrations (10^5, 10^6). For each pathogen, correlation values obtained among the three replicate experiments, each using three fruit, were as follows: *C. gloeosporioides* (r^2 = 0.50838^2, P = 0.0001), *P. perseae* (r^2 = 0.24433^2, P = 0.0001), *Pestalotiopsis versicolor* (r^2 = –0.28866^2, P = 0.0001), *D. aromatica* (r^2 = –0.11446^2, P = 0.0768), *F. solani* (r^2 = 0.11780^2, P = 0.0715), and *T. pseudotrichia* (r^2 = 0.11972^2, P = 0.0641). When data from the six pathogens evaluated on Fuerte fruit in artificial inoculation studies were combined and means were plotted for the different antagonist and pathogen concentrations, a typical dose–response relationship was found (Fig. 8), with greater inhibition of lesion development associated with increasing antagonist concentration and decreasing pathogen spore levels.

Correlation coefficients obtained between the two disease evaluation criteria, lesion surface area and disease severity rating, for the various pathogens were highly significant (*C. gloeosporioides* [r^2 = 0.82548^2], *P. perseae* [r^2 = 0.81600^2], *Pestalotiopsis versicolor* [r^2 = 0.41838^2], *D. aromatica* [r^2 = 0.77296^2], *F. solani* [r^2 = 0.23649^2], *T. pseudotrichia* [r^2 = 0.56416^2]), all at P = 0.0001. Data from the artificial inoculation experiment with Fuerte fruit correlated well with those obtained on Edranol fruit (r^2 = 0.08907^2, P = 0.0292) and are therefore not presented.

When applied in Tag-wax, *B. subtilis* significantly reduced external anthracnose and SE ratings, except for the 10^5 cells ml^{-1} concentration, which did not reduce external SE (Table 2). Antagonist concentrations of 10^8 and 10^7 cells ml^{-1} significantly reduced internal anthracnose, whereas the

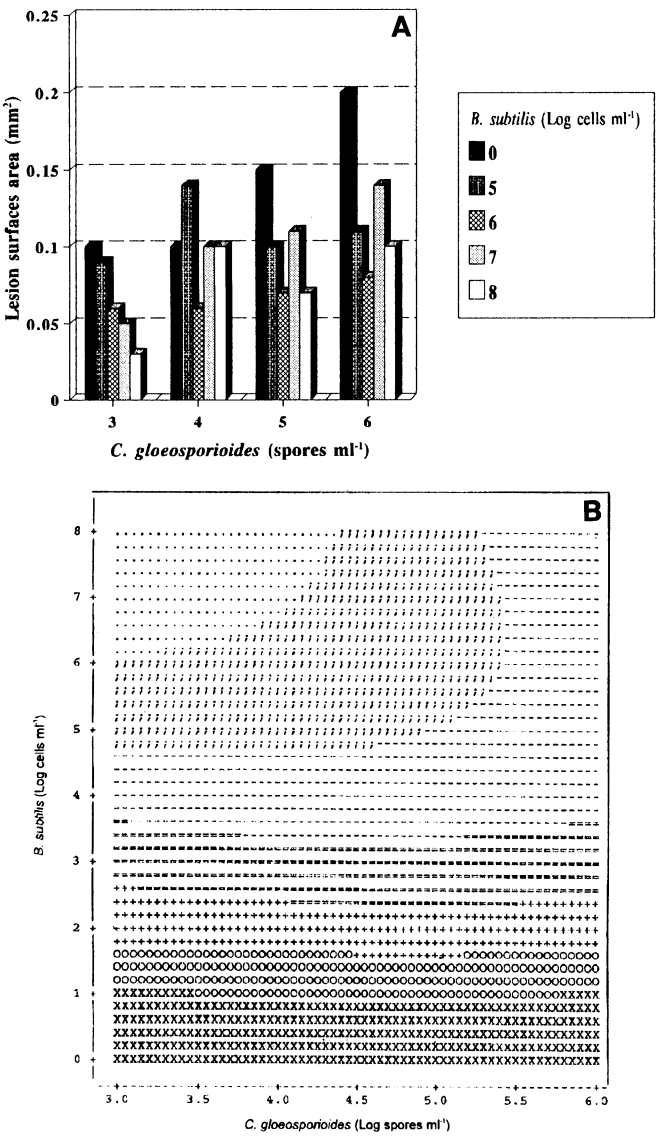

Fig. 2. Effect of *Bacillus subtilis* on **(A)** observed (arcsine transformed) and **(B)** predicted lesion development caused by *Colletotrichum gloeosporioides* on Fuerte avocado fruit using a checkerboard-type titration method. Y = 4.35 + 0.0000024 × pat + 0.000002 × ant + 0.01170461 × pat^2 – 0.1289556 × ant – 0.778269 × ant × pat + 0.10541 × ant^2 × pat + 0.0827780 × ant × pat^2 – 0.006545 × ant^2 × pat^2 – 0.002222 × ant × pat^3 – 0.00184 × ant^3 × pat + 0.00000850 × ant^3 × pat^3. R^2 = 0.69, where ant = *B. subtilis* concentration and pat = *C. gloeosporioides* spore concentrations. Model significant at P = 0.0001. Symbols represent the predicted disease values, with negative to small values indicating low level and larger values indicating an increase in disease severity. If $-4 \le Y < -2$, symbol = ... ; if $-2 \le Y < -1$, symbol = ;;; ; if $-1 \le Y < 0$, symbol = --- ; if $0 \le Y < 1$, symbol = === ; if $1 \le Y < 2$, symbol = +++ ; if $2 \le Y < 3$, symbol = ooo ; if $3 \le Y < 4$, symbol = xxx.

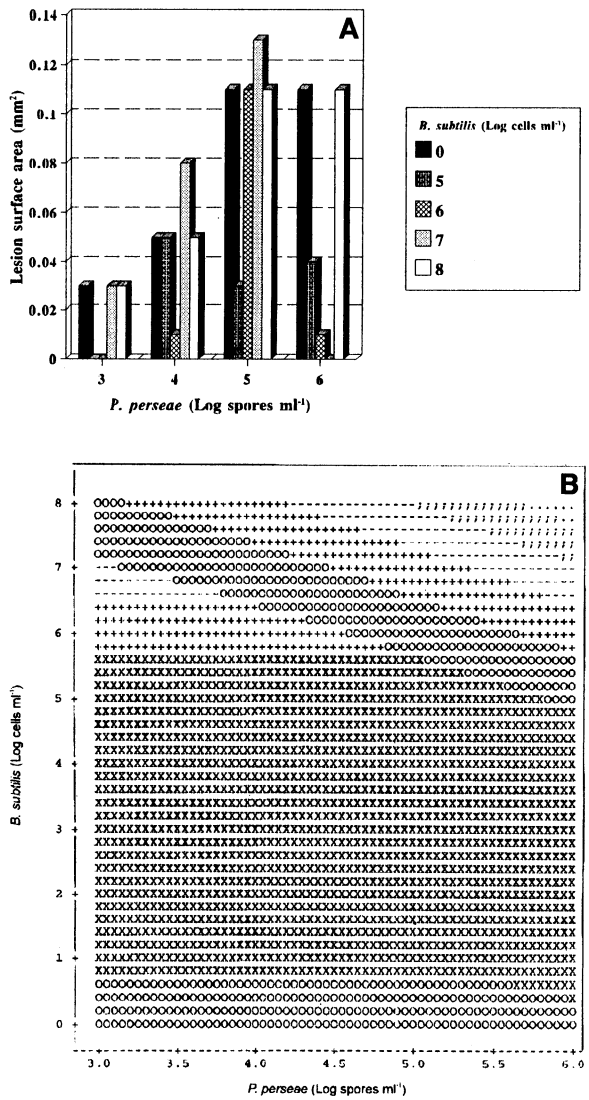

Fig. 3. Effect of *Bacillus subtilis* on **(A)** observed (arcsine transformed) and **(B)** predicted lesion development caused by *Phomopsis perseae* on Fuerte avocado fruit using a checkerboard-type titration technique. Y = 13.61 – 0.00000127 × pat – 0.00000003 × ant + 0.02329792 × pat^2 – 0.0028685 × ant^2 + 0.376575 × ant × pat – 0.04919 × ant^2 × pat – 0.0575955 × ant × pat^2 + 0.004297 × ant^2 × pat^2 + 0.0013183 × ant × pat^3 + 0.000993 × ant^3 × pat + 0.00000612 × ant^3 × pat^3. R^2 = 0.97, where ant = *B. subtilis* concentration and pat = *P. perseae* spore concentrations. Model significant at P = 0.0001. Symbols represent the predicted disease values, with negative to small values indicating low level and larger values indicating an increase in disease severity. If $11.5 \le Y < 12$, symbol = ... ; if $12 \le Y < 12.5$, symbol = ;;; ; if $12.5 \le Y < 13$, symbol = --- ; if $13 \le Y < 13.5$, symbol = +++ ; if $13.5 \le Y < 14$, symbol = ooo ; if $14.5 \le Y < 15$, symbol = xxx.

lowest two concentrations (10^6 and 10^5 cells ml^{-1}) were significantly more effective against DCC. Only the 10^7 cells ml^{-1} antagonist concentration decreased internal SE. However, all *B. subtilis* concentrations tested controlled postharvest diseases of avocado equally well when the mean of each disease was compared with the untreated control.

DISCUSSION

In vitro evaluation of bacteria as potential antagonists from a variety of agricultural crops has been described (4,6,12, 22,37). This is, however, the first report in which bacteria from avocado leaf and fruit surfaces have been isolated and their antagonistic potential evaluated against avocado fruit pathogens. The most inhibitory bacteria belonged to the genus *Bacillus. B. subtilis*, *B. cereus,* and *B. licheniformis* were previously shown to be antagonistic in vitro against a range of plant pathogens (6,11,22,27,28,32), but their antifungal activity against *D. aromatica, T. pseudotrichia, Pestalotiopsis versicolor,* and *P. perseae* was demonstrated for the first time here. In addition, *B. subtilis* controlled artificial infections of *C. gloeosporioides, P. perseae, Pestalotiopsis versicolor, D. aromatica, F. solani,* and *T. pseudotrichia* and natural infections of postharvest pathogens on avocado fruit. Such a wide spectrum of activity is uncommon in biological control. For instance, Janisiewicz (13) used *Acremonium breve* (Sukapure & Thirumalachar) W. Gouws and *Pseudomonas* sp. in combination against blue mold (caused by *Penicillium expansum* Link ex Gray) and gray mold (caused by *Botrytis cinerea* Pers ex Nocca and Balb), because neither antagonist was effective against both pathogens on Golden Delicious apples. Similarly, McLaughlin et al. (24) succeeded in

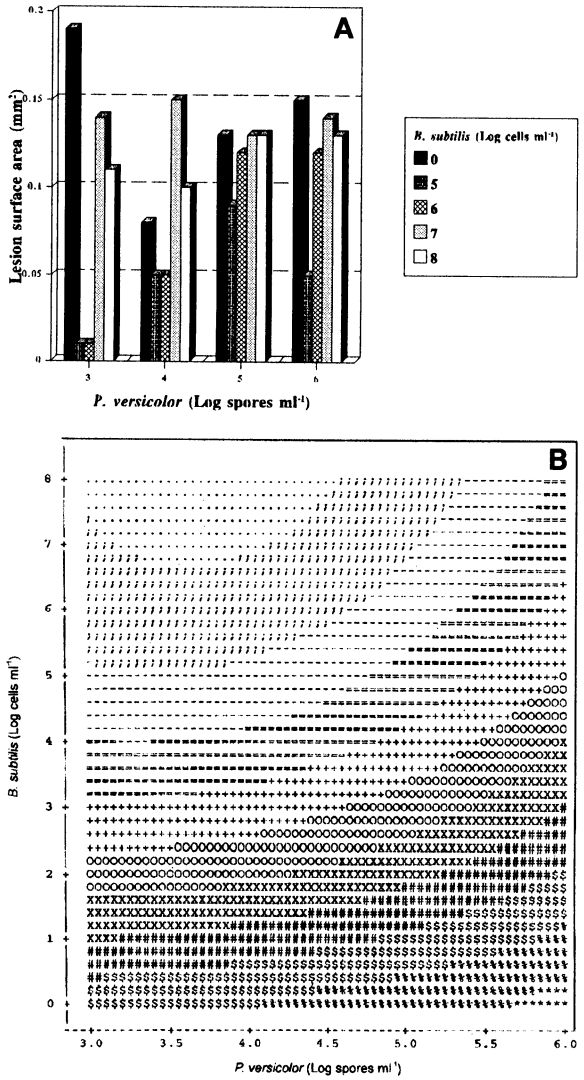

Fig. 4. Effect of *Bacillus subtilis* on **(A)** observed (arcsine transformed) and **(B)** predicted lesion development caused by *Pestalotiopsis versicolor* on Fuerte avocado fruit using a checkerboard-type titration method. $Y = 1.95 - 0.00000239 \times pat - 0.00000003 \times ant + 0.03259573 \times pat^2 + 0.0224452 \times ant^2 - 0.551677 \times ant \times pat + 0.02147 \times ant^2 \times pat + 0.0917683 \times ant \times pat^2 - 0.004252 \times ant^2 \times pat^2 - 0.0026123 \times ant \times pat^3 + 0.000149 \times ant^3 \times pat + 0.00000617 \times ant^3 \times pat^3$. $R^2 = 0.70$, where ant = *B. subtilis* concentration and pat = *P. versicolor* spore concentrations. Model significant at $P = 0.0001$. Symbols represent the predicted disease values, with negative to small values indicating low level and larger values indicating an increase in disease severity. If $-2 \le Y < 1.5$, symbol = ... ; if $-1.5 \le Y < -1$, symbol = ;;; ; if $-1 \le Y < -0.5$, symbol = --- ; if $-0.5 \le Y < 0$, symbol = === ; if $0 \le Y < 0.5$, symbol = +++ ; if $0.5 \le Y < 1$, symbol = ooo ; if $1 \le Y < 1.5$, symbol = xxx ; if $1.5 \le Y < 2$, symbol = ### ; if $2 \le Y < 2.5$, symbol = $$$; if $2.5 \le Y < 3$, symbol = %%% ; if $3 \le Y < 3.5$, symbol = ***.

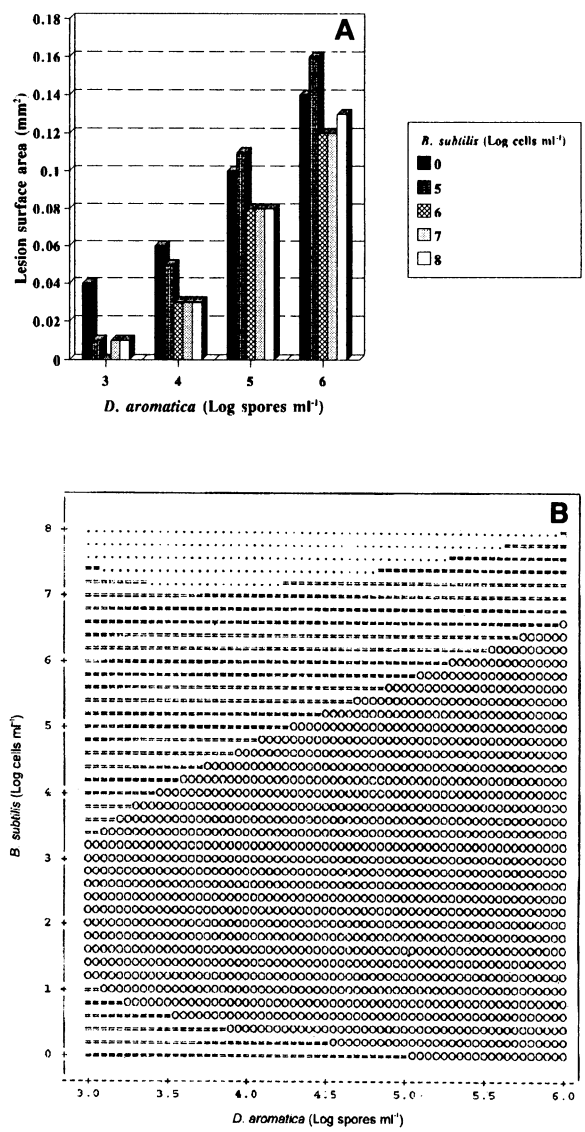

Fig. 5. Effect of *Bacillus subtilis* on **(A)** observed (arcsine transformed) and **(B)** predicted lesion development caused by *Dothiorella aromatica* on Fuerte avocado fruit using a checkerboard-type titration technique. $Y = 3.8 + 0.00000081 \times pat - 0.00000003 \times ant + 0.00798823 \times pat^2 + 0.0228812 \times ant^2 + 0.006651 \times ant \times pat - 0.02475 \times ant^2 \times pat + 0.0196589 \times ant \times pat^2 - 0.000059 \times ant^2 \times pat^2 - 0.0006715 \times ant \times pat^3 + 0.000859 \times ant^3 \times pat - 0.00000011 \times ant^3 \times pat^3$. $R^2 = 0.74$, where ant = *B. subtilis* concentration and pat = *D. aromatica* spore concentrations. Model significant at $P = 0.0001$. Symbols represent the predicted disease values, with negative to small values indicating low level and larger values indicating an increase in disease severity. If $3 \le Y < 3.4$, symbol = ... ; if $3.4 \le Y < 4$, symbol = === ; if $4 \le Y < 5$, symbol = ooo.

reducing artificial and natural infections of *Rhizopus stolonifer* (Ehrenb. ex Link) Lind on grape with the yeasts *Kloeckera apiculata* (Reess emend. Klöcker) Juncke and *Pichia guilliermondii* Wickerham, while decay caused by *Aspergillus niger* Tiegh. remained unaffected.

An important attribute of a successful biocontrol agent is the ability to be efficient at low concentrations (38). *B. subtilis* (B246) conformed to this prerequisite by being generally effective against the various avocado postharvest pathogens and/or diseases at the lowest concentration (10^5 cells ml^{-1}), both in laboratory tests and in the packinghouse. Droby et al. (9) reported that a high concentration (10^9 cfu ml^{-1}) of *Debaromyces hansenii* (Zopf) Van Rij was necessary for control of green mold of citrus caused by *Penicillium digitatum* (Pers. ex Fr.) Sacc. and that application of lower concentrations of the antagonist resulted in increased infection. Control of *D. aromatica* was achieved by all antagonist concentrations, but only at the lower pathogen challenge levels (10^3 and 10^4 spores ml^{-1}). In analogy, Janisiewicz and Roitman (16) reported that high concentrations of *B. cinerea* could not be controlled by high antagonist levels. In the present investigation, more effective control of *P. perseae, Pestalotiopsis versicolor,* and *T. pseudotrichia* was not achieved by increasing antagonist concentration. This is contrary to previous findings of increased control with increased antagonist concentrations (12,28). However, *B. subtilis* applied at 10^8 cells ml^{-1} prevented symptom development by *F. solani* and *T. pseudotrichia*, resulting in very little lesion expansion, irrespective of the inoculum level. Although Janisiewicz and Roitman (16) reported similar "no lesion development" at the highest *P. cepacia* concentration, this was only achieved at pathogen challenge levels of 10^3 and 10^4 conidia ml^{-1} for *B. cinerea* or *Penicillium expansum*. In general, however, increased control of avocado postharvest diseases tended to be associated with increasing antagonist concentrations and decreasing

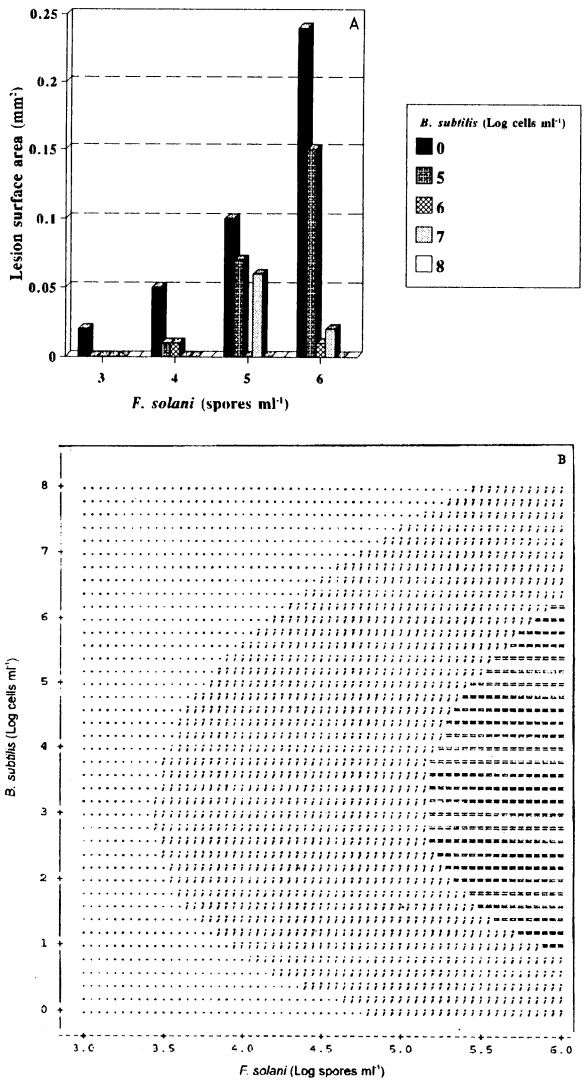

Fig. 6. Effect of *Bacillus subtilis* on **(A)** observed (arcsine transformed) and **(B)** predicted lesion development caused by *Fusarium solani* on Fuerte avocado fruit using a checkerboard-type titration technique. $Y = 1.30 + 0.00000178 \times pat - 0.00000005 \times ant + 0.07471290 \times pat^2 + 0.0696034 \times ant^2 + 0.051705 \times ant \times pat - 0.05780 \times ant^2 \times pat + 0.040210 \times ant \times pat^2 - 0.000523 \times ant^2 \times pat^2 - 0.0009647 \times ant \times pat^3 + 0.00201 \times ant^3 \times pat - 0.00000063 \times ant^3 \times pat^3$. $R^2 = 0.97$, where ant = *B. subtilis* concentration and pat = *F. solani* spore concentrations. Model significant at $P = 0.0001$. Symbols represent the predicted disease values, with negative to small values indicating low level and larger values indicating an increase in disease severity. If $-4 \leq Y < 3$, symbol = ... ; if $3 \leq Y < 5$, symbol = ;;; ; if $5 \leq Y < 8$, symbol = ===.

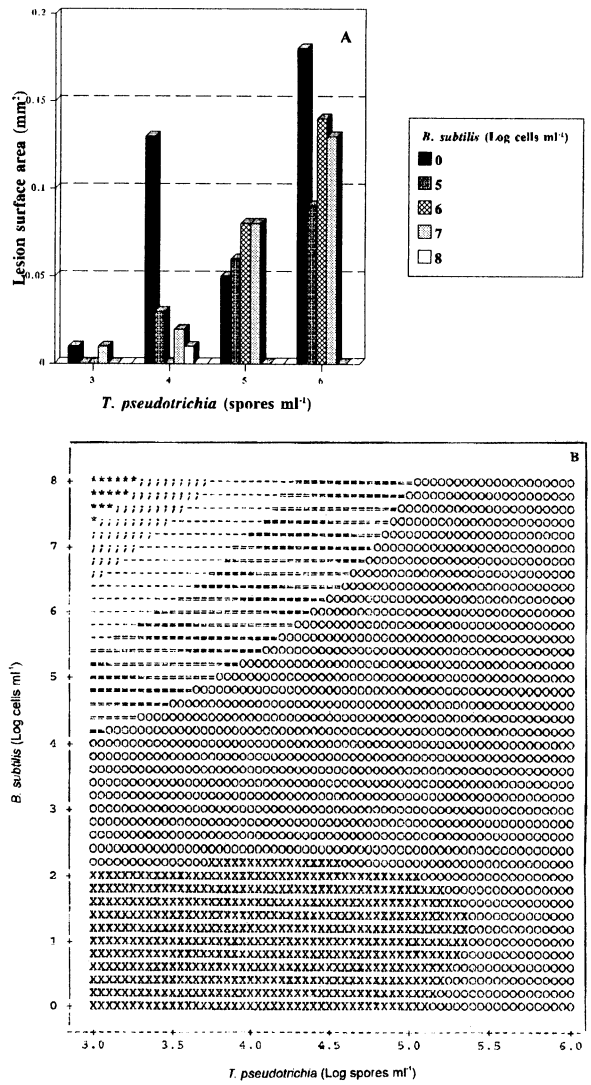

Fig. 7. Effect of *Bacillus subtilis* on **(A)** observed (arcsine transformed) and **(B)** predicted lesion development caused by *Thyronectria pseudotrichia* on Fuerte avocado fruit using a checkerboard-type titration technique. $Y = 8.616 + 0.0000189 \times pat + 0.0000003 \times ant + 0.01464961 \times pat^2 + 0.3105637 \times ant^2 - 0.077063 \times ant \times pat - 0.09922 \times ant^2 \times pat + 0.0296028 \times ant \times pat^2 + 0.009042 \times ant^2 \times pat^2 - 0.003331 \times ant \times pat^3 - 0.00019 \times ant^3 \times pat - 0.00000888 \times ant^3 \times pat^3$. $R^2 = 0.89$, where ant = *B. subtilis* concentration and pat = *T. pseudotrichia* spore concentrations. Model significant at $P = 0.0001$. Symbols represent the predicted disease values, with negative to small values indicating low level and larger values indicating an increase in disease severity. If $8 \leq Y < 9$, symbol = *** ; if $9 \leq Y < 10$, symbol = ;;; ; if $10 \leq Y < 11$, symbol = --- ; if $11 \leq Y < 12$, symbol = === ; if $12 \leq Y < 13$, symbol = ooo ; if $13 \leq Y < 14$, symbol = xxx.

spore challenge levels of the pathogen, a phenomenon that is common in biological control (9,12,16,28).

As most avocado postharvest pathogens occur in the orchard as latent skin infections (26), the significant control achieved in the packinghouse has demonstrated *B. subtilis* to be effective against established infections. Moreover, few reports refer to biocontrol of postharvest diseases incited by natural infections. Pusey et al. (29) incorporated *B. subtilis* into commercial wax for control of brown rot of peaches after artificial inoculation of fruit with *Monilia fructicola* (Wint.) Honey, whereas Chalutz et al. (4) succeeded in reducing natural infections of *Penicillium italicum* Wehmer and *P. digitatum* on grapefruit by *Pichia guilliermondii* on a semicommercial scale in the packinghouse. In the present investigation, disease control was still evident after 21 days in the laboratory and after 35 days in the packinghouse treatment, when the respective experiments were terminated. This contrasts with previous biocontrol studies, which mainly report temporary effects (9,25).

The checkerboard-type titration assay used here proved to be a useful screening procedure for establishing optimal antagonist concentrations on avocado, as opposed to the single-fruit assays utilized in other studies (14,25,29). Advantages of the checkerboard technique in the avocado system include a reduction in the number of fruit required for the various pathogen–antagonist concentrations and the facilitation of evaluating this range of concentrations on the same fruit, thereby reducing variation in response among replicates.

This investigation has shown that the avocado phyllo- and fructoplane is inhabited by various bacterial epiphytes inhibitory toward postharvest pathogens infecting the crop. *Bacillus* spp. comprised a major component of the microflora and some of them, especially *B. subtilis*, proved to be highly effective as inoculant biocontrol agents against postharvest diseases. The next phase of this investigation would obviously involve further field and packinghouse evaluations of the antagonist, and also elucidation of its mode of action. The latter aspect is of crucial importance, since a commercial partner for marketing the organism would be reluctant to participate if it produces a metabolite unsafe for human consumption.

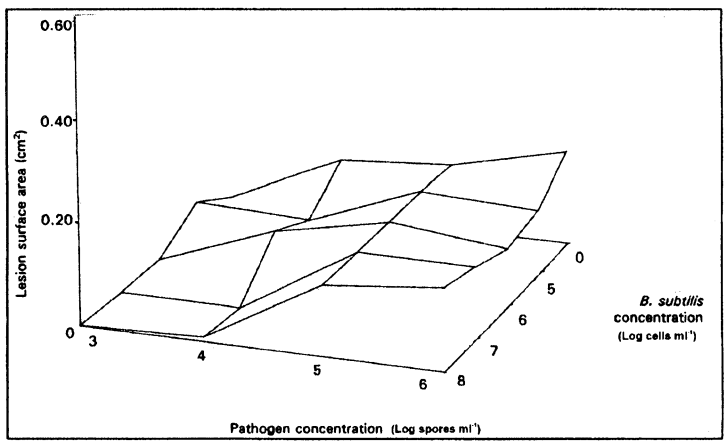

Fig. 8. Effect of increasing *Bacillus subtilis* concentrations on lesion surface area resulting from infection with increasing concentrations of avocado postharvest pathogens. Data points represent mean lesion surface area from three experiments and the mean of six avocado postharvest pathogens at each infection concentration.

Table 2. Effect of various concentrations of *Bacillus subtilis* applied in Tag-wax on postharvest diseases of Hass avocado fruit at Westfalia Estate

B. subtilis cells ml^{-1}	Disease severity					Treatment means[y]
	External evaluations		Internal evaluations			
	Anthracnose	SE[x]	Anthracnose	SE[x]	DCC[x]	
0	1.02 a[z]	0.10 a	2.09 a	1.43 a	0.36 a	1.00 a
10^8	0.34 bc	0.01 b	1.51 b	1.39 a	0.17 ab	0.69 b
10^7	0.40 bc	0.01 b	1.40 b	0.93 b	0.25 ab	0.60 b
10^6	0.21 c	0.01 b	1.62 ab	1.17 ab	0.04 b	0.61 b
10^5	0.61 b	0.03 ab	1.85 ab	1.10 ab	0.10 b	0.74 b
F value	6.13	2.41	2.18	2.22	2.82	2.89
PR > *F*	0.0001	0.0480	0.0702	0.0655	0.0243	0.0217

[x] SE = stem-end rot; DCC = *Dothiorella/Colletotrichum* fruit rot complex.

[y] Treatment mean of external and internal postharvest diseases combined for each treatment.

[z] Means within columns followed by the same letter do not differ significantly ($P = 0.05$) according to Duncan's multiple range test. Values indicate mean disease severity at ready-to-eat ripeness. Fruit was evaluated on a 0 to 10 scale, 0 being healthy and 10 indicating entire fruit decay (8).

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