Chapter 2

COMPARISON OF POSTHARVEST *BACILLUS* APPLICATIONS, FUNGICIDAL AND INTEGRATED TREATMENTS FOR CONTROL OF CITRUS POSTHARVEST DISEASES

ABSTRACT

Bacillus spp., previously isolated from subtropical plants and fruit, were evaluated *in vitro, in vivo* and in packhouse dip or wax treatments for control of citrus postharvest diseases caused by *Alternaria citri, Colletotrichum gloeosporioides, Lasiodiplodia theobromae, Geotrichum citri-aurantii, Penicillium digitatum, P. italicum* and *Trichoderma viride*. *Bacillus subtilis* (B246) inhibited *in vitro* growth of the citrus postharvest pathogens most effectively, while *Bacillus licheniformis* (B251) and *B. subtilis* (B248) were the most effective antagonists *in vivo* against *P. digitatum*. In packhouse experiments an integrated control, consisting of antagonist combined with application of quarter-strength solutions of the standard concentrations of guazatine, imazalil, thiabendazole, was included along with a standard chemical treatment. Although treatment with antagonists B246 and *B. licheniformis* (B254) reduced the percentage of fruit infected, the reduction was less than that achieved with the standard chemical treatment. Integrated treatments were as effective as the chemical treatments, implicating the integrated approach as an alternative disease control strategy requiring reduced rates of fungicides.

INTRODUCTION

Alternaria citri Ellis & N. Pierce (*Alternaria* rot), *Aspergillus niger* Tiegh. (*Aspergillus* rot), *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (anthracnose), *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl. (stem-end rot), *Geotrichum citri-aurantii* (Ferraris) E.E. Butler (sour rot), *Penicillium digitatum* (Pers.: Fr.) Sacc. (green mould), *Penicillium italicum* Wehmer (blue mould), *Rhizopus stolonifer* (Ehrenb.: Fr.) Vuill. (*Rhizopus* rot), *Trichoderma viride* Pers.: Fr. (*Trichoderma* rot) (Brown & Eckert, 1989; Eckert & Brown, 1989) and *Penicillium ulaiense* Hsieh, Su & Tzean (whisker mould) (Holmes *et al.*, 1994), are the major pathogens causing citrus postharvest decay. Control of these pathogens relies mainly on treatment with fungicides such as guazatine, imazalil and thiabendazole (Shachnai & Barash, 1982; Eckert & Ogawa, 1985; Pelser, 1988). However, the use of fungicides is becoming increasingly restricted because of their potential detrimental effect on the environment and human health (Norman, 1988; Huang *et al.*, 1995). In addition, development of pathogen resistance is further restricting use of these fungicides (Bancroft *et al.*, 1984; Bus *et al.*, 1991; Eckert *et al.*, 1994). This, and regulatory restrictions by the US Environmental Protection Agency regarding the use of fungicides (Couey, 1989), necessitate development of new technologies for control of postharvest diseases as an alternative to fungicides (Van Staden, 1994).

Biological control using microbial antagonists is considered a desirable and rapidly developing alternative, either on its own or as part of an integrated control strategy to reduce fungicide input (Teixidó *et al.*, 1998). Antagonists have been reported to control postharvest diseases in many fruit commodities e.g. avocado (Korsten *et al.*, 1995), apple (Janisiewicz *et al.*, 1998), peach (Pusey *et al.*, 1988), mango (Koomen & Jeffries, 1993, De Villiers & Korsten, 1996), litchi (Korsten *et al.*, 1993), and pear (Janisiewicz & Criof, 1992; Sugar & Spotts, 1999). Currently, a number of commercial products have been registered and are available for control of postharvest diseases, including Aspire (*Candida oleophila* strain I-182; Ecogen Inc., Langhorne, PA) and Bio-Save 110 and 111 (*Pseudomonas syringae* strains ESC 10 and ESC 11, EcoScience Corp., Worcester, MA) (Cook *et al.*, 1996; Teixidó *et al.*, 1998).

According to Chalutz & Wilson (1990), an early observation on biological control of citrus postharvest diseases was reported by Gutter & Littauer in 1953, who isolated *Bacillus subtilis* from citrus fruit that inhibited the growth of citrus pathogens. More recently, several other antagonists have been reported to control citrus postharvest diseases, including *Candida* spp. (Arras, 1996; Arras *et al.*, 1997, 1999; Droby *et al.*, 1997), *Pichia* spp. (Droby *et al.*, 1997) and *Pseudomonas* spp. (Huang *et al.*, 1995; Bull *et al.*, 1997; Smilanick *et al.*, 1999). This study reports the effect of *Bacillus* spp., alone or integrated with fungicides, on postharvest decay of Valencia oranges.

MATERIALS AND METHODS

In vitro screening of antagonists

Antagonists (obtained from L. Korsten, Department of Microbiology and Plant Pathology, University of Pretoria, Pretoria, SA) were screened for inhibition of citrus postharvest pathogens *A. citri, C. gloeosporioides, L. theobromae, G. citri-aurantii, P. digitatum, P. italicum* and *T. viride* (obtained from K. Lesar, Capespan Citrus Centre, Nelspruit, SA). Antagonists included *Bacillus subtilis* (isolates B246 and B248 from avocado), *B. licheniformis* (isolates B250 and B251 from mango and isolate B254 from litchi leaf surfaces), *B. stearothermophilus* (isolate B252 from litchi), *B. megaterium* (isolate B253 from litchi) and *B. cereus* (isolates B247 and B249 from avocado). These *Bacillus* antagonists were rpeviously shown to be effective against pathogens associated with the subtropical fruit from which they were isolated (Korsten *et al.*, 1992, 1993; Korsten, 1993).

The dual culture technique (Porter, 1924; Skidmore, 1976) was used to test antagonism on nutrient agar (Biolab, Johannesburg, SA). Antagonists were streaked 30 mm from the 5-mm diameter pathogen disc. Three replicates were included for each antagonist-pathogen combination and controls consisted of fungal discs without antagonist streaks. Mean percentage pathogen inhibition was determined from the formula of Skidmore (1976): (r - C) x 100 / C, where r = the radius from the middle of the pathogen colony, on the side facing the antagonist, and C = growth from the middle to the edge of the pathogen on control plates. Data was statistically analysed using an analysis of variance and Duncan's multiple range test to

separate means (P = 0.05).

In vivo screening of antagonists

Antagonists (B246, B248, B250, B251, B254) evaluated in *in vitro* screening assays were grown in 500 ml Erlenmeyer flasks containing 250 ml Standard 1 nutrient broth (STD 1) (Biolab). After 48 h shake-incubation (rotary shaker, 67 rpm) at 28°C, cells were harvested by centrifugation for 10 min at 8000 g. The resulting pellet was dissolved in quarter-strength Ringer solution (Merck, Johannesburg) to obtain a concentration series of 10^6, 10^7, 10^8 and 10^9 cells ml^{-1} using a Petroff-Hausser counting chamber. Citrus postharvest pathogen, *P. digitatum*, was cultured for 14 days on potato-dextrose agar (Biolab) at 25°C under a near-UV light source. Conidia were harvested in quarter-strength Ringer solution, counted with a haemacytometer and serially diluted to obtain a concentration series ranging from 10^3 to 10^6 conidia ml^{-1}.

Valencia orange fruit were used to determine the most effective antagonist concentration according to the checkerboard-titration technique used by Korsten *et al.* (1995). Fruit were surface-sterilised by dipping in 70 % ethanol and left to air-dry. Squares (5 mm x 5 mm) were drawn on one side of the fruit with waterproof ink to give five vertical and five horizontal rows forming a checkerboard pattern of 25 squares. The surface of the fruit in each square was prick-wounded centrally to a depth of 5 mm with a sterile 1 mm-diameter inoculation needle. Twenty microlitres of each *P. digitatum* concentration was applied to a square resulting in horizontal rows having the same conidial concentration (Fig. 1). Fruit were left to air-dry and after 24 h, 20 µl of the appropriate antagonist was applied to a square using the same concentration in vertical rows. The last row received 20 µl quarter-strength Ringer solution only. Three fruit were used for each antagonist-*P. digitatum* combination. Inoculated fruit were randomly packed in cardboard boxes lined with moistened cotton wool to maintain humidity. Boxes were stored at 25 °C, and disease development was scored after seven days according to an arbitrary scale of 0 and 1, where 0 = no disease development or slight browning around the edge of the inoculation point, and 1 = fungal growth within the wound or necrotic enlargement spreading from the wound.

Compatibility of antagonists with chemicals

Antagonists selected for their strong inhibitory action *in vitro* against the citrus postharvest pathogens were tested for compatibility with fungicides and a wax applied commercially in citrus packhouses, using a modified paper-disc method (Thornberry, 1950). Antagonists were incorporated in STD1 agar . Paper discs (Whatman, 5mm) saturated with one of the following chemicals, guazatine (1000 ppm and 250 ppm) (Decotine) (Waltiernon Dormas (Pty) Ltd., Johannesburg); thiabendazole (1000 ppm and 250 ppm) (Tecto) (Logos Agrowett (Pty) Ltd., Johannesburg) 2,4-D (500 ppm and 125 ppm) (Agrihold, Silverton, Pretoria, SA); imazalil (500 ppm and 125 ppm) (Agrihold) and sodium-orthophenyl-phenol (SOPP) (1000 ppm and 250 ppm) (Agrihold) were placed on the agar. Controls consisted of discs saturated with sterile water. Citrashine wax (Plaaschem, Houghton, SA) was tested similarly to method used to test the chemicals. Plates were incubated at 25 °C for 24 and 48 hours after which inhibition or stimulation zones were recorded using the scale 0 = no growth; 1 = > 5 mm inhibition zone around paper disc; 2 = < 5 mm inhibition zone around paper disc and 3 = paper disc overgrown.

Postharvest experiments

Efficacy of antagonists on their own, integrated with quarter-strength chemicals and commercial chemical treatments were evaluated on Valencia orange fruit, obtained from Letaba Estates (Tzaneen, Northern Province, SA) (Tables 1, 2 and 3). Antagonists were prepared according to Korsten *et al.* (1989).

Fruit used in all treatments were submerged in a commercial chlorine bath (100 ppm) followed by high-pressure water spray (40 bar) before treatment. Fruit were mechanically injured subsequently by rolling them over a strip of drawing pins (Fig. 2d, e). This caused 7-9 pinpricks up to 3 mm deep on each fruit. Wounded fruit were artificially inoculated by dipping the fruit for 3 min in a drum containing 25 l inoculum (Fig. 2c, f). Inoculum was prepared by macerating fruit naturaly infected with postharvest pathogens (*A. citri, C. gloeosporioides, L. theobromae, G. citri-aurantii, P. digitatum, P. italicum* and *T. viride).* The homogenate was filtered through four layers of cheesecloth (Fig. 2a, b). Fungal spores in the filtrate were counted using a haemacytometer and the filtrate was diluted in tap water to a concentration of 10^2, 10^3 or 10^5 *Penicillium* conidia ml^{-1}. Fruit were incubated overnight at 25 °C and treated the

following day.

Fruit were dipped in 50 l containers with tap water containing either the antagonist or chemical (2,4-D, guazatine, imazalil and TBZ in experiment 1; or guazatine and imazalil in experiments 2 and 3) or both (Fig. 2g). After fungicide dip treatment in experiments 2 and 3, fruit were waxed with Citrashine supplemented with TBZ. Integrated treatments were dipped in water containing quarter-strength of the standard fungicide concentrations (imazalil and guazatine) and subsequently waxed with Citrashine supplemented with antagonist and quarter-strength TBZ. In the last two experiments (2 and 3) all antagonists were applied in wax. Each treatment consisted of 800 fruit.

After dip and wax treatments, fruit were left to air-dry, packed in cartons (80 fruit per box) (Fig. 2h) and sealed. Cartons were covered with black plastic and stored in darkness at room temperature. The plastic was removed after four days and the fruit stored for an additional 14 days. Sound fruit per carton were counted and percentages computed. Data were analysed statistically using ANOVA, and Student's t-least significant differences were calculated to determine treatment differences (P = 0.05).

RESULTS

In vitro screening of antagonists

Differences in the degree of fungal inhibition were evident between the nine antagonists evaluated (Table 4). Overall, *B. subtilis* (isolate B246) inhibited growth of the postharvest pathogens the most, although not significantly more than *B. licheniformis* (isolate B250) and *B. megaterium* (isolate B253). Growth of *C. gloeosporioides* was affected less than that of *A. citri*, *G. citri-aurantii*, *P. digitatum* and *P. italicum*.

In vivo screening of antagonists

At pathogen concentrations of 10^4 conidia.ml^{-1} and lower, all antagonists at all concentrations

prevented infection by *P. digitatum* except for isolate B246 which was only effective at a concentration of 10^9 cells ml^{-1} for a 10^4 conidia ml^{-1} inoculum and at 10^7 cells ml^{-1} and higher for a 10^3 conidia ml^{-1} inoculum (Table 5). Concentrations of 10^8 cells ml^{-1} and higher of B248 and B251 were necessary to prevent infection at an inoculum level of 10^5 conidia ml^{-1}, while 10^9 cells ml^{-1} of B250 and B254 were required for effective control. None of the antagonists could prevent infection by *P. digitatum* at an inoculum level of 10^6 conidia ml^{-1}.

Antagonist compatibility with chemicals

None of the chemicals except Citrashine were fully compatible with the antagonists, when tested at commercial concentrations (Table 6). However, when tested at quarter- strength concentrations only SOPP totally inhibited the growth of all antagonists.

Postharvest experiments

Experiment 1: Only the chemical and integrated treatments significantly decreased postharvest decay, although not significantly more so than B248 dip against natural infection and B254 dip against both natural and artificially infected fruit (Fig. 3, 4).

Experiment 2: All treatments significantly increased percentage sound fruit compared to the wax control. The chemical, quarter-strength chemical and integrated treatments were the most effective treatments (Fig. 5). Applying B246, B250 and B254 as a mixture in wax was less effective than wax treatment with B246 and B254 individually, but not than treatment with B250 wax.

Experiment 3: The chemical, integrated and quarter-strength treatments increased percentage sound fruit from less than 10 % to more than 90 % (Fig. 6).

DISCUSSION

Bacillus subtilis is a known antagonist of fruit pathogens, including those of citrus (Korsten, 1993). This, however, is the first report of the antagonistic ability of *B. licheniformis*, *B.*

megaterium and *B. stearotermophilus* against citrus postharvest pathogens.

The most effective antagonist *in vitro* was *B. subtilus* (B246). However, high concentrations of the bacterium were required to control *P. digitatum in vivo*. Control could also be achieved only at lower inoculum concentrations of the pathogen. Corresponding findings were reported by Huang *et al.* (1992), regarding the control of *P. digitatum* with *Bacillus pumilus*.

With the exception of B246, all antagonists tested *in vivo*, were capable of controlling *P.digitaum* inoculated at 10^5 conidia ml^{-1} when applied at concentrations of 10^9 cells ml^{-1}. *Bacillus licheniformis* (B254) and *B. subtilis* (B248), however, achieved control at this pathogen inoculum level using concentrations of only 10^8 cells ml^{-1}. In general, enhanced disease control was associated with increased antagonist concentration or a decrease in the pathogen inoculum level, a phenomenon which is well described in biocontrol (Pusey & Wilson, 1984; Janisiewicz & Roitman, 1988; Korsten *et al.*, 1995). The disparity between *in vitro* and *in vivo* results highlights the importance of using both methods in a screening programme (Utkhede *et al.*, 1986; Chalutz & Wilson, 1990).

According to Chalutz & Wilson (1990), packhouse antagonist treatments must be as effective and provide consistent control comparable to that achieved with chemical treatments. Although some antagonist treatments reduced decay compared to the control in both experiments 1 and 2, they were not as effective or consistent as chemical treatments. Shachnai *et al.* (1996) and Droby et *al.* (1998) experienced a similar phenomenon in packhouse experiments with *Candida oleophila* Montrecher. However, when the yeast was applied in combination with SOPP and low concentrations of TBZ, control equivalent to chemical treatment was achieved. Antagonists used in the present study were tolerant to low concentrations of imazalil, 2.4 D and TBZ. In packhouse experiments, the efficacy of these antagonists was enhanced when used in combination with low concentrations of the above chemicals and disease control achieved was comparable with that of standard chemical treatments.

Application method is another important factor in antagonist treatments of fruit, which has to be compatible with existing packhouse procedures (McGuire, 1994). Pusey *et al.* (1986, 1988) and Korsten (1993) incorporate the antagonist *B. subtilis* into fruit waxes used

commercially in peach and avocado packhouses, repectively. In this study, Citrashine wax provided a viable mean of applying antagonists to fruit. Variability recorded in this study could have resulted from several factors related to the initial quality of the fruit, susceptibility of the fruit to infection, time elapsed between picking and treatment, and initial inoculum density (Droby *et al.*, 1993). Inoculum density in the final experiment was 100 times higher than in the second experiment, thus implicating that inoculum pressure could have been a reason for the ineffectiveness of the antagonists. Applying antagonists in wax had the added advantage of requiring relative small quantities of antagonist (1 l wax for 52 cartons) and being less time- consuming to apply.

As indicated above, control of postharvest diseases depends largely on the inoculum level of the pathogen. Furthermore, effectiveness of antagonist treatments relies on the successful establishment of the antagonist at wound sites prior to pathogen challenge (Janisiewicz & Roitmann, 1988; Huang *et al.*, 1992). In this study, however, the pathogen inoculum was applied prior to antagonist treatment. Artificial infection used in this investigation simulated field conditions, as pathogens usually infect fruit preharvest through wounds, as well as postharvest through wounds and natural openings (Sommer, 1982; Brown & Eckert, 1989; Eckert & Brown, 1989), before antagonist application in the packhouse. According Korsten (1993), using biocontrol agents preharvestly to reduce the incidence of postharvest diseases, leads to a persistent reduction in pathogen inoculum. As antagonists used in this study proved capable of controlling postharvest pathogens *in vitro* and *in vivo*, establishing them preharvest may provide a more effective control option.

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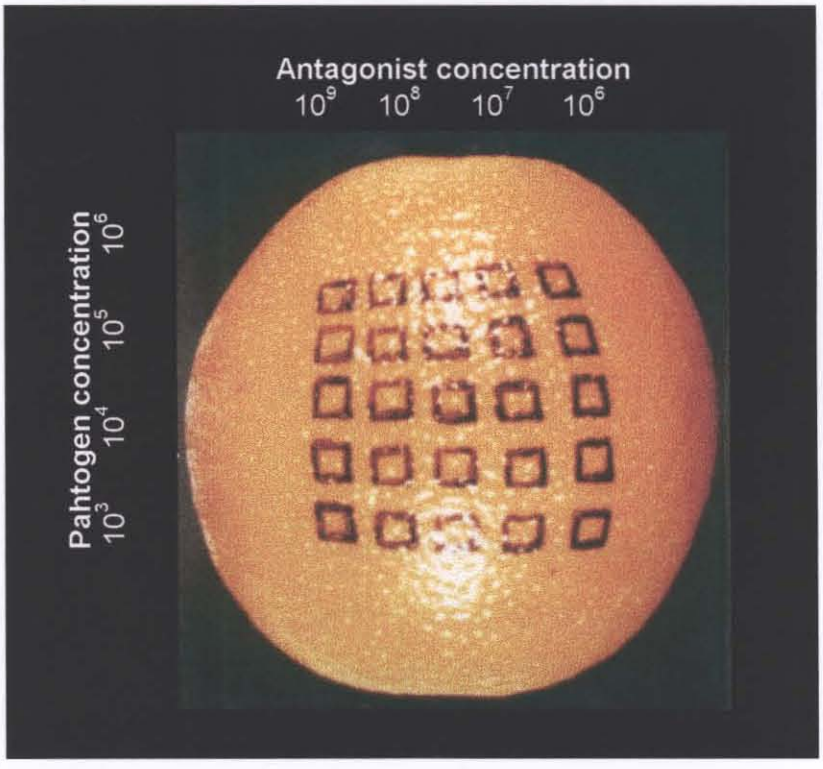


Fig. 1 Checkerboard-titration technique with different antagonist and pathogen concentrations on Valencia orange fruit.

Table 1 Postharvest treatment of Valencia orange fruit for evaluation of biological, integrated and chemical control of citrus postharvest diseases (Experiment 1)

Code	Treatment	Concentration of antagonist (cells ml^{-1}) or chemical (ppm)	Application time (min)
1	Control[y]	-	-
2	B246 dip[y]	1.36×10^7	7
3	B248 dip[y]	7.36×10^7	7
4	B250 dip[y]	6.60×10^7	7
5	B254 dip[y]	1.43×10^7	7
6	B250 wax[y]	1.05×10^7	-
7	Integrated[y]	guazatine (250); 2,4-D (125);	
		thiabendazole (250); imazalil (125);	3
		followed by B254 (1.43×10^7)	7
8	Chemical[z]	guazatine (1000); 2,4-D (500);	
		thiabendazole (1000); imazalil (500)	3

[y] Treatments consisted of fruit both naturally and artificially infected. Fruit were artificially infected with a mixed inoculum of *Alternaria citri*, *Colletotrichum gloeosporioides*, *Geotrichum citri-aurantii*, *Lasiodiplodia theobromae*, *Penicillium digitatum*, *P. italicum* and *Trichoderma viride*, containing 1.1×10^2 *Penicillium* conidia ml^{-1}.

[z] Treatment involved only naturally infected fruit.

Table 2 Postharvest treatment of Valencia orange fruit for evaluation of biological, integrated and chemical control of citrus postharvest diseases (Experiment 2)

Code	Treatment [z]	Concentration of antagonist (cells ml^{-1}) or chemical (ppm)	Application time (min)
1	Wax control	-	-
2	B246 wax	7.32×10^7	-
3	B254 wax	6.84×10^7	-
4	B250 wax	7.44×10^7	-
5	Wax antagonist mixture	B246, B250, B254 (1.20×10^7)	-
6	Integrated	guazatine (250) and imazalil (125) dip followed by thiabendazole (250) and B246 (7.32×10^7) wax	3
7	¼ Chemical	guazatine (250) and imazalil (125) dip followed by thiabendazole (250) wax	3
8	Chemical	guazatine (1000) and imazalil (500) dip followed by thiabendazole (1000) wax	3

[z] Fruit of all treatments were mechanically damaged and artificially infected with a mixed inoculum of *Alternaria citri*, *Colletotrichum gloeosporioides*, *Geotrichum citri-aurantii*, *Lasiodiplodia theobromae*, *Penicillium digitatum*, *P. italicum* and *Trichoderma viride*, containing 1.2×10^3 *Penicillium* conidia ml^{-1}.

Table 3 Postharvest treatment of Valencia orange fruit for evaluation of biological, integrated and chemical control of citrus postharvest diseases (Experiment 3)

Code	Treatment [z]	Concentration of antagonist (cells ml^{-1}) or chemical (ppm)	Application time (min)
1	Wax control	-	
2	B246 wax	2.53×10^7	
3	B254 wax	5.61×10^7	
4	Wax antagonist mixture	B246, B250, B254 (1.54×10^7)	
5	Integrated	guazatine (250) and imazalil (125) dip followed by thiabendazole(250) and B246 (2.53×10^7) wax	3
6	1/4 Chemical	guazatine (250) and imazalil (125) dip followed by thiabendazole (250) wax	3
7	Chemical	guazatine (1000) and imazalil (500) dip followed by thiabendazole (1000) wax	3

[z] Fruit of all treatments were mechanically damaged and artificially infected with a mixed inoculum of *Alternaria citri*, *Colletotrichum gloeosporioides*, *Geotrichum citri-aurantii*, *Lasiodiplodia theobromae*, *Penicillium digitatum*, *P. italicum* and *Trichoderma viride*, containing 2.6×10^5 *Penicillium* conidia ml^{-1}.

Table 4 Comparison of *Bacillus* spp. for their inhibitory effect on the *in vitro* growth of seven postharvest citrus pathogens

Antagonist	Code	Percentage inhibition [x]							MEAN[y]	Pr > F
		P1	P2	P3	P4	P5	P6	P7		
Bacillus cereus	B247	49 aA	30 aB	42 aAB	32 bB	12 cC	18 cC	25 bBC	30 c	0.0001
B. cereus	B249	48 aA	24 aC	38 aAB	46 abA	32 bcB	26 bcBC	23 bC	34 bc	0.0006
B. licheniformis	B250	40 abcAB	34 aB	32 abB	42 abAB	52 aA	50 aA	42 aAB	42 ab	0.0056
B. licheniformis	B251	32 eB	24 aC	28 abBC	34 bB	56 aA	54 aA	20 bC	35 bc	0.0001
B. licheniformis	B254	39 bcdAB	28 aB	31 abB	33 bB	40 bA	42 abA	36 abAB	36 bc	0.0053
B. megaterium	B253	36 deB	28 aC	27 abC	36 bB	46 abA	43 abA	42 aA	37 abc	0.0078
B. stearothermophilus	B252	36 deB	30 aBC	34 abB	34 bB	33 bcB	35 bAB	40 aA	35 bc	0.0346
B. subtilis	B246	45 aB	33 aC	31 abC	54 aA	58 aA	56 aA	43 aB	46 a	0.0003
B. subtilis	B248	40 abcAB	28 aC	30 abBC	33 bB	46 abA	43 abAB	22 bC	35 bc	0.0006
MEAN[z]		37 AB	29 C	33 BC	38 AB	42 A	41 A	33 BC		0.0001
Pr > F		0.0021	0.3696	0.0602	0.0395	0.0008	0.0052	0.0463	0.0001	

[x] Determined according to Skidmore (1976); P1 = *Alternaria citri*, P2 = *Colletotrichum gloeosporioides*, P3 = *Lasiodiplodia theobromae*, P4 = *Geotrichum citri-aurantii*, P5 = *Penicillium digitatum*, P6 = *P. italicum* and P7 = *Trichoderma viride*; values within columns (lower case) and rows (upper case) followed by the same letter do not differ significantly according to Duncan's multiple range test (P = 0.05).

[y] Mean percentage inhibition of pathogens P1 – P7.

[z] Mean percentage inhibition of individual pathogens by all *Bacillus* spp.

Table 5 Comparison of the quantitative relationship between different *Bacillus* spp. and *Penicillium digitatum* concentrations according to the checkerboard-titration technique

Antagonist	Code	Concentration (cells ml^{-1})	Disease severity[z]			
			10^3	10^4	10^5	10^6
Bacillus subtilis	B246	10^0	1	1	1	1
		10^6	1	1	1	1
		10^7	0	1	1	1
		10^8	0	1	1	1
		10^9	0	0	1	1
B. subtilis	B248	10^0	1	1	1	1
		10^6	0	0	1	1
		10^7	0	0	1	1
		10^8	0	0	0	1
		10^9	0	0	0	1
B. licheniformis	B250	10^0	1	1	1	1
		10^6	0	0	1	1
		10^7	0	0	1	1
		10^8	0	0	1	1
		10^9	0	0	0	1
B. licheniformis	B251	10^0	1	1	1	1
		10^6	0	0	1	1
		10^7	0	0	1	1
		10^8	0	0	0	1
		10^9	0	0	0	1
B. licheniformis	B254	10^0	1	1	1	1
		10^6	0	0	1	1
		10^7	0	0	1	1
		10^8	0	0	1	1
		10^9	0	0	0	1

[z] Disease development at different inoculum concentrations (conidia ml^{-1}) of *P. digitatum*, where 0 = no disease development or slight browning around the edge of the inoculation point, and 1 = fungal growth within the wound or necrotic enlargement spreading from the wound.

Table 6 Compatibility of *Bacillus* spp. with chemicals commercially used in citrus packhouses

		Antagonist growth[z] at different chemical concentrations (ppm)										
Antagonist	**Code**	2,4-D		guazatine		imazalil		sodium-orthophenyl-phenol		thiabendazole		Citrashine wax
		500	125	1000	250	500	125	1000	250	1000	250	
B. licheniformis	B250	1[z]	2	1	2	0	1	0	0	1	2	3
B. licheniformis	B254	1	2	1	2	0	1	0	0	1	2	3
B. megaterium	B253	1	2	1	2	0	1	0	0	1	2	3
B. subtilis	B246	1	2	1	2	0	1	0	0	1	2	3
B. subtilis	B248	1	2	1	2	0	1	0	0	1	2	3

[z] 0 = no growth; 1 = > 5mm inhibition zone around paper disc; 2 = < 5mm inhibition zone around paper disc; and 3 = antagonist grows over paper disc.

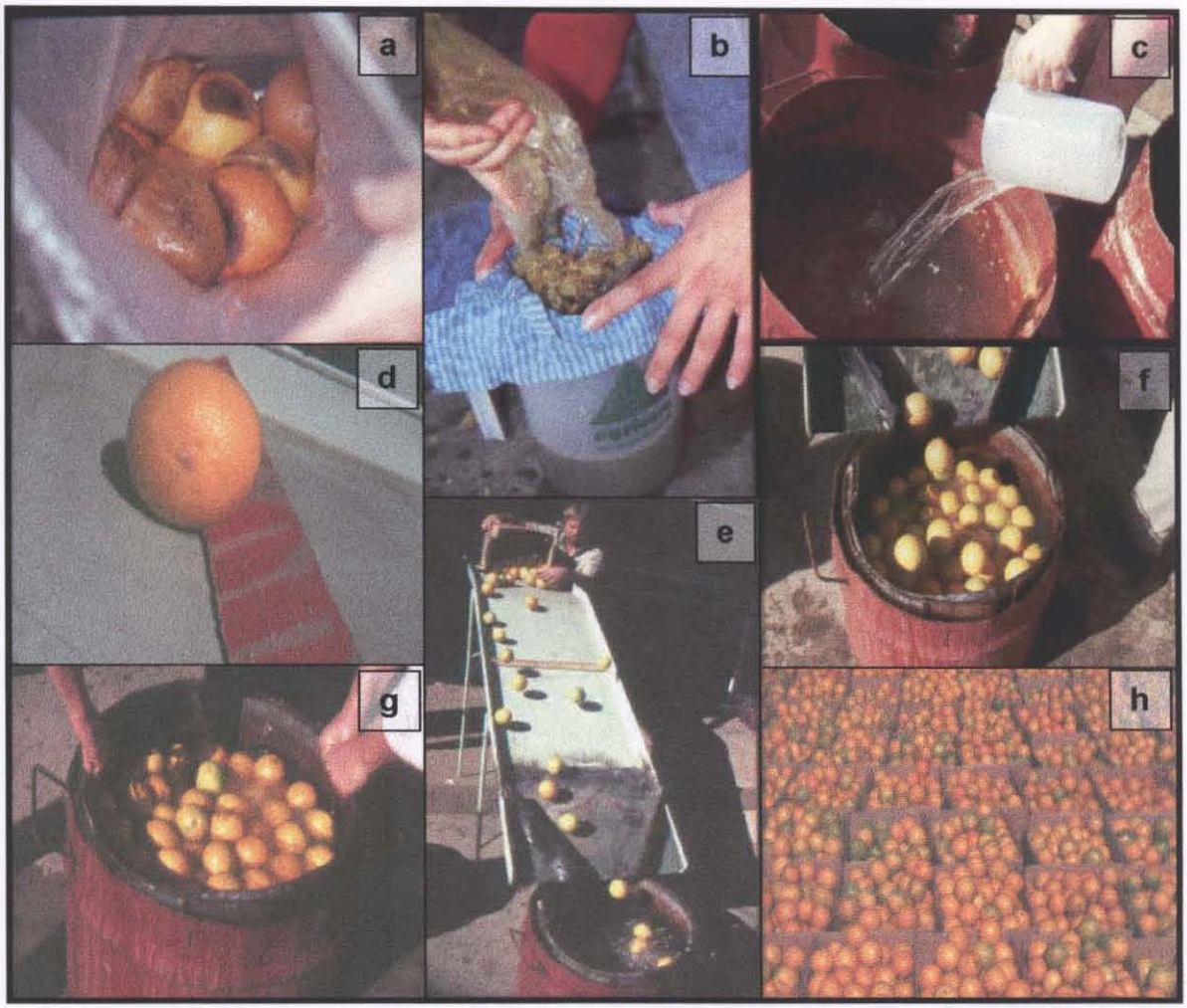


Fig. 2 Artificial inoculation and treatment of Valencia orange fruit.

a Source of inoculum (fruit infected with postharvest pathogens).

b Preparation of inoculum (macerate fruit and strain through cheese cloth).

c Adjust macerated and strained pathogen suspension by adding water to required concentration.

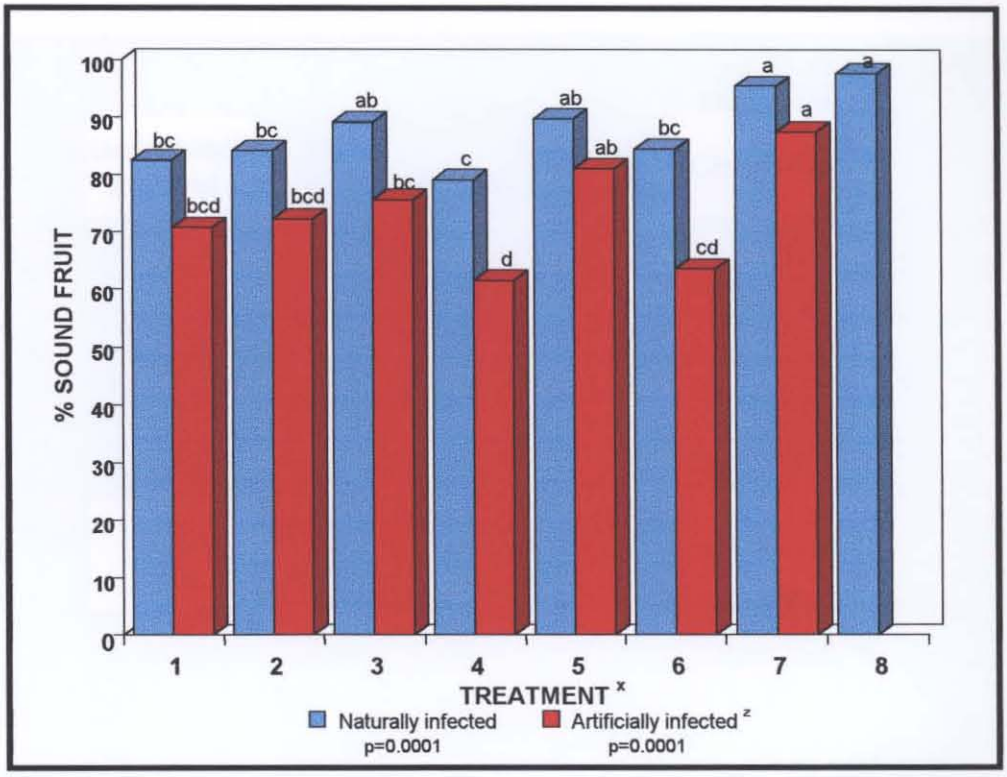
d Close-up of drawing pin strip used to injure fruit (7-9 pinpricks up to 3 mm deep per fruit).

e Process of mechanical injury and artificial inoculation of fruit.

f Close-up of artificial inoculation for 3 min.

g Dip treatment of fruit.

h Packing of treated fruit prior to storage.



Bars not sharing a common letter differ significantly according to Student's t-LSD (P=0.05).

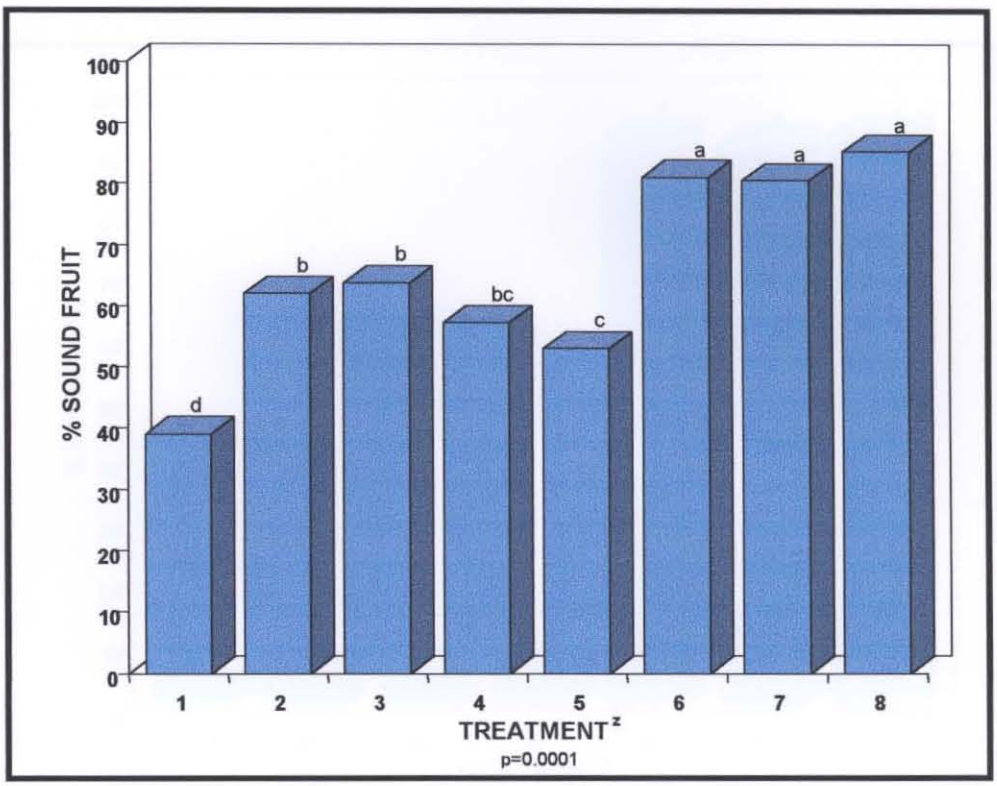
[x] See Table 1 for treatment descriptions: 1 = untreated control; 2 = B246 dip; 3 = B248 dip; 4 = B250 dip; 5 = B254 dip; 6 = B250 wax; 7 = Integrated; 8 = chemical treatment

[z] Treatments consisted of mechanically injured fruit which were artificially inoculated by dipping in 25 l inoculum of *Alternaria citri*, *Colletotrichum gloeosporioides*, *Geotrichum citri-aurantii*, *Lasiodiplodia theobromae*, *Penicillium digitatum*, *P. italicum* and *Trichoderma viride*, containing 1.1×10^2 *Penicillium* conidia ml^{-1}. Fruit were incubated overnight and treated the following day.

Fig. 3 Effect of postharvest *Bacillus* spp., chemical and integrated dip and wax treatments on postharvest deterioration of Valencia orange fruit (Experiment 1).

Fig. 4 Effect of postharvest treatment of Valencia orange fruit on the development of decay (Experiment 1).

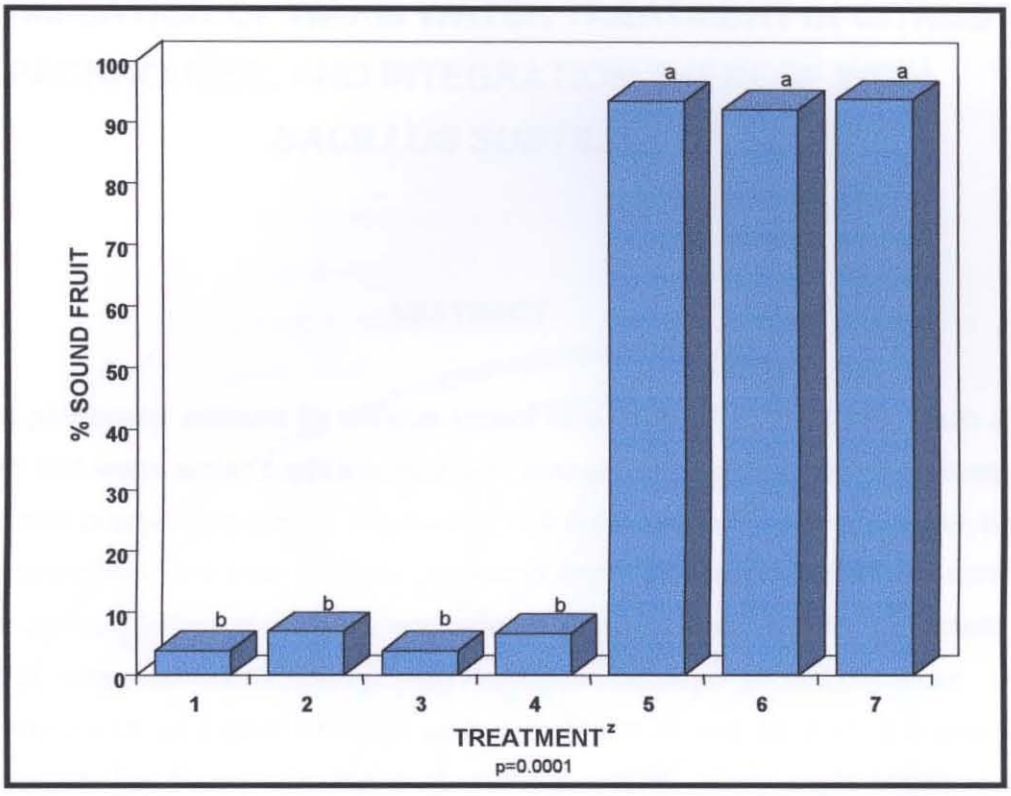
Treatments included: untreated control; chemical control consisting of guazatine (1000ppm); 2,4-D (500ppm); thiabendazole (1000ppm); imazalil (500ppm) 3 min dip; B254 (1.43×10^7 cells ml^{-1}) 7 min dip; and an integrated treatment consisting of guazatine (250ppm); 2,4-D (125ppm); thiabendazole (250ppm); imazalil (125ppm) 3 min dip followed by B254 (1.43×10^7 cells ml^{-1}) 7 min dip.



Bars not sharing a common letter differ significantly according to Student's t-LSD (P=0.05).

[z] See Table 2 for treatment descriptions: 1 = wax control; 2 = B246 wax; 3 = B254 wax; 4 = B250 wax; 5 = wax antagonist mixture; 6 = Integrated; 7 = ¼ strength chemical; 8 = chemical treatment. Treatments consisted of mechanically injured fruit which were artificially inoculated by dipping in 25 l inoculum of *Alternaria citri*, *Colletotrichum gloeosporioides*, *Geotrichum citri-aurantii*, *Lasiodiplodia theobromae*, *Penicillium digitatum*, *P. italicum* and *Trichoderma viride*, containing 1.2 x 10^3 *Penicillium* conidia ml^{-1}. Fruit were incubated overnight and treated the following day.

Fig. 5 Effect of postharvest *Bacillus* spp., chemical and integrated dip and wax treatments on postharvest deterioration of Valenicia orange fruit (Experiment 2).



Bars not sharing a common letter differ significantly according to Student's t-LSD (P=0.05).

[z] See Table 3 for treatment descriptions: 1 = wax control; 2 = B246 wax; 3 = B254 wax; 4 = wax antagonist mixture; 5 = Integrated; 6 = ¼ strength chemical; and 7 = chemical treatment. Treatments consisted of mechanically injured fruit which were artificially inoculated by dipping in 25 l inoculum of *Alternaria citri*, *Colletotrichum gloeosporioides*, *Geotrichum citri-aurantii*, *Lasiodiplodia theobromae*, *Penicillium digitatum*, *P. italicum* and *Trichoderma viride*, containing 2.6 x 10^5 *Penicillium* conidia ml^{-1}. Fruit were incubated overnight and treated the following day.

Fig. 6 Effect of postharvest *Bacillus* spp., chemical and integrated dip and wax treatments on postharvest deterioration of Valenicia orange fruit (Experiment 3).