Use of *Xanthomonas campestris* pv. *vesicatoria* to Evaluate Surface Disinfectants for Canker Quarantine Treatment of Citrus Fruit

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ABSTRACT

Brown, G. E., and Schubert, T. S. 1987. Use of *Xanthomonas campestris* pv. *vesicatoria* to evaluate surface disinfectants for canker quarantine treatment of citrus fruit. Plant Disease 71:319-323.

Florida citrus packinghouses are required by quarantine regulations to use chlorine or sodium orthophenylphenate (SOPP) to surface-sanitize asymptomatic fruit to eradicate a bacterial disease currently classified as citrus canker (*Xanthomonas campestris* pv. *citri*). Treatments with chlorine or SOPP in soak or spray applications require exposures for 2 and 1 min, respectively, whereas soap formulations of SOPP applied during washing require 45 sec. In this study, applications of chlorine or SOPP during washing for 30 sec were as effective as the longer exposures currently required. Wash applications of dual quaternary ammonium compounds, formulations of chlorine dioxide, or peracetic acid for a similar time were equally effective. *X. campestris* pv. *vesicatoria* was used in these studies as the assay bacterium because it responded similarly to the canker bacterium in in vitro disinfectant tests and could be used outside of quarantine facilities.

Additional key words: *Citrus sinensis*, postharvest pathology

A citrus bacterial disease caused by a bacterium identified as *Xanthomonas campestris* (*X. c.*) pv. *citri* was discovered in nursery stock in central Florida in August 1984 (17). This was the first report of the disease in Florida since citrus bacterial canker was eradicated in 1933 (5). Eradication of this new outbreak is being attempted following guidelines developed and enforced by federal and state quarantine agencies (1). To prevent the inadvertent spread of viable bacterial cells on surfaces of asymptomatic fruit, all citrus fruit packed in Florida and sold for fresh consumption must be surface-sterilized during the packing process. Treatments with chlorine or sodium orthophenylphenate (SOPP), a fungicide used routinely for many years to control citrus fruit decays (7), are currently approved for fruit sanitation (1).

Use of the chlorine treatment was approved on the basis of studies conducted in Japan with the Asiatic form of the citrus canker organism (13). SOPP was approved after efficacy was established in studies conducted in quarantine facilities in Florida. With chlorine (150–250 μg/ml free residual, pH 6.0–7.5), unwashed fruit must be thoroughly wetted for 2 min with the solution with or without 0.05% (v/v) nonionic surfactant by soaking fruit in a tank or by spraying fruit after dumping (1). In many Florida packinghouses, the 2-min exposure to chlorine can only be obtained by applying the spray immediately after the dump and continuing periodic sprays to the fruit to maintain continuous wetting as it is conveyed through the presizer and washer. SOPP is applied at concentrations of 1.86–2.00%, those commonly used for decay control (7). Formulations applied as a spray or drench must wet the fruit for 1 min. If the material is applied during washing in soap formulations, the fruit must be treated for 45 sec.

Florida Agricultural Experiment Station Journal Series No. 7360.

Accepted for publication 31 October 1986 (submitted for electronic processing).

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Exposure times required for fruit disinfection are based on data developed from applications of chlorine (13) or SOPP to unwashed fruit. Certain characteristics of chlorine and SOPP, such as corrosiveness and phytotoxicity, respectively, are undesirable and may be alleviated with shorter exposure times. These materials, chlorine dioxide, quaternary ammonium compounds, and peracetic acid were evaluated in this study in spray applications to unwashed fruit or in applications during washing.

MATERIALS AND METHODS

Inoculum. All in vitro studies with the isolates of *X. c.* pv. *citri* were conducted in the quarantine facilities of the Florida Department of Agriculture and Consumer Services, Division of Plant Industry, at Gainesville. An isolate of *X. c.* pv. *vesicatoria* was used at Lake Alfred for studies with fruit disinfection.

Isolates of the two bacteria were maintained on Difco potato-dextrose agar or nutrient agar for production of inoculum. Cultures 1 wk old or less were used for all studies. Cells were removed from culture plates, suspended in sterile tap water, and adjusted to a concentration of 10^8 colony-forming units (cfu) per milliliter (absorbance of 0.3 at 600 μm) with a spectrophotometer and diluted to the desired concentrations.

Fruit treatments. Fruit (*Citrus sinensis* cv. Valencia) were harvested at one time from one grove, wrapped in polyethylene film (3), and stored at 5 C until used in the various trials. After the fruit were atomized with bacterial cells (10^6 cfu/ml), they were held in plastic bags or covered plastic pans near 100% relative humidity and treated with disinfectant within 15 min. Evaluations of the disinfectants applied as sprays before washing were conducted by atomizing the test material on eight infested fruit, each supported on a 15.2-cm wooden applicator stick inserted at the stem end. Fruit treated with disinfectant for 2 min were sprayed initially and again after 1 min to maintain continual wetness. Opposite surfaces of each fruit were swabbed after 1 or 2 min with separate sterile cotton swabs, and the contents were streaked on a single plate of selective media.

An evaluation of each disinfectant applied during washing was made by washing eight fruit individually in the disinfectant for 30 sec on 30.5-cm-wide polyethylene brushes (30 wraps per 30.5 cm) rotating at 90 rpm. The disinfectants were prepared, poured into a 946-ml plastic hand-spray bottle, and atomized on individual infested fruit. After the brushes were first saturated with the disinfectant, each fruit was then sprayed with the disinfectant and immediately placed on the rotating brushes. The disinfectant was continually atomized on the fruit during washing for 20 sec. Runoff from the fruit and brushes was collected on selective media during the last 5 sec of this period. The fruit was washed an additional 10 sec (total 30 sec) without applying more disinfectant. After 30 sec, the fruit was removed aseptically from the brushes with plastic gloves disinfested with 70% ethanol. Opposite surfaces of the fruit were swabbed and the contents plated as described previously. Surfaces of each of the two brushes used to wash the fruit also were swabbed and platings done in a similar fashion to check for viable cells. Between each evaluation of individual fruit, the washer brushes were thoroughly cleaned with a spray of tap water and metal bars mounted beneath the brushes were forced against them to remove excess water and remaining residue of disinfectant. The brushes were then sprayed with chlorine (200 μg/ml free residual, pH 7), which eradicated surviving bacteria, thoroughly washed again to remove chlorine, and dewatered again with the metal bars.

Media. Medium selective for *X. c.* pv. *vesicatoria* (11) was modified to inactivate the various disinfectants. Lecithin (1 g/L), used to inactivate phenol and quaternary ammonium compounds (10,16), was melted and emulsified in boiling water. The components of the selective medium (11) were dissolved in the emulsion, and the agar was added and melted before autoclaving. Sodium thiosulfate (0.4 g/L) was added to the selective medium (11) after autoclaving to inactivate chlorine, chlorine dioxide, and peracetic acid. Inactivation of the disinfectants was ascertained by coating surfaces of the hardened selective medium with the respective disinfectants with a cotton swab. Cells of *X. c.* pv. *vesicatoria* (10^6 cfu/ml) were streaked on the dried surface, and ensuing growth confirmed inactivation of the disinfectant compared with lack of growth on the control.

Lethality of the disinfectants within a minimum exposure time of 30 sec was observed with a modification of the membrane filter technique (14). Membranes supporting 5×10^2 cfu of *X. c.* pv. *vesicatoria* were covered with 20 ml of disinfectant, except with formulations of SOPP, where only 5 ml was used because of slow filtration. The disinfectants were removed by filtration and cells were then washed twice with 10 ml of Difco potato-dextrose broth to remove residues of disinfectant. Four washes were used to

Table 1. Comparison of the eradicant activity of disinfectants applied to the surface of Valencia oranges infested with *Xanthomonas campestris* pv. *vesicatoria*

Disinfectant	Time of exposure (min)	Rate (μg/ml)	Rating[v]
Control[w]	2	...	3.0 a[x]
Chlorine	2	200	1.3 b
Chlorine dioxide	2	200	1.4 b
Quaternary ammonium[y]	1	1,000	1.1 b
SOPP[z]	1	20,000	0.1 c
Peracetic acid	1	200	0.1 c

[v] Bacterial cells were removed from the surfaces of unwashed fruit 1 or 2 min after spraying the fruit with disinfectant. Ratings of 0, 1, 2, or 3 represent 0–5, 6–100, 101–200, and 201+ colony-forming units, respectively, per plate and are the means of eight observations.
[w] Fruit were treated with water.
[x] Values followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.
[y] Bioserve formulation of quaternary ammonium.
[z] Freshgard 5 formulation of sodium orthophenylphenate.

Table 2. Reduction in populations of *Xanthomonas campestris* pv. *vesicatoria* applied to Valencia oranges and recovered after application of chlorine or chlorine dioxide during washing for 30 sec

Disinfectant	Rate (μg/ml)	Rating[x] R	F	B
Control	...	2.0 ± 0.3	2.0 ± 0.2	2.4 ± 0.3
Chlorine[y]	150	0.1 ± 0.1	1.0 ± 0.3	0.1 ± 0.1
	200	0.3 ± 0.3	0.8 ± 0.2	0.1 ± 0.1
Chlorine dioxide	50	0.1 ± 0.1	1.0 ± 0.3	0.4 ± 0.2
	100	0.1 ± 0.1	0.8 ± 0.2	0.1 ± 0.1
	200	0.0 ± 0.0	0.5 ± 0.2	0.1 ± 0.1
Alcide	1:200:1[z]	0.3 ± 0.2	2.5 ± 0.3	0.8 ± 0.2
	1:100:1	0.0 ± 0.0	2.0 ± 0.1	0.3 ± 0.2
	1:90:1	0.3 ± 0.2	0.0 ± 0.0	0.6 ± 0.2
	1:80:1	0.1 ± 0.1	0.0 ± 0.0	0.6 ± 0.2

[x] Ratings of 0, 1, 2, or 3 represent 0–5, 6–100, 101–200, and 201+ colony-forming units, respectively, per plate and are the means and standard errors of eight observations. Cells were recovered in the runoff (R) and from fruit (F) and brush (B) surfaces.
[y] Concentrations of chlorine were significantly ($P = 0.05$) correlated with recovery of bacteria from fruit surfaces ($r^2 = 0.997$).
[z] Ratio of 1 part base (sodium chlorite):specified parts water:1 part activator (lactic acid).

remove quaternary ammonium compounds. After treatment, the membranes with cells were placed on selective media containing appropriate inactivators and plates were incubated 2–3 days at 28 C, then observed for bacterial growth.

Sensitivity to the various disinfectants of one isolate of *X. c.* pv. *vesicatoria* and two Florida isolates of *X. c.* pv. *citri* used in this study was compared in vitro. Cell concentrations were diluted, and 0.1 ml was added to 9.9 ml of each disinfectant and exposed for 2, 4, or 6 min. After each period of exposure, 0.1 ml with about 100–150 cfu was spread evenly with a sterile glass rod over the surface of a plate of selective media containing inhibitor. The plates were incubated for 3–4 days at 28 C and observed for bacterial colonies.

A rapid, simple method for enumeration of bacterial survival in numerous treatments of a relatively large number of fruit was developed to test the efficacy of the various disinfectants. After streaking cells from brush runoff or from fruit or brush surfaces onto the selective media, the plates were incubated at 28 C. After 3–4 days, each plate was numerically rated 0, 1, 2, or 3 on the basis of the number of cfu that developed on each plate. These ratings represented about 0–5, 6–100, 101–200, and 201+ cfu, respectively, per plate.

Disinfectants. Dual quaternary ammonium formulations evaluated in these tests were *n*-alkyl (60% C14, 30% C15, 5% C12, and 5% C18) dimethyl benzyl ammonium chlorides and *n*-alkyl (68% C12 and 32% C14) dimethyl ethyl benzyl ammonium chlorides (Gallex 900, Galloway Chemical Division, Clearwater, FL; Bioserve, Bioserve, Inc., Boca Raton, FL). Chlorine compounds were chlorine prepared from 5.25% sodium hypochlorite and chlorine dioxide prepared from muriatic acid and sodium chlorite (4) (Clow Corporation, Water Management Division, Jacksonville, FL) and from lactic acid and sodium chlorite (Alcide Corporation, Farmingdale, NY). Phenol compounds were Freshgard 5, 24% SOPP (anhydrous) (FMC Corporation, Lakeland, FL); Dow-Hex Concentrate, 14.11% SOPP (anhydrous) and SOPP soap, 12.6% SOPP (anhydrous) (American Machinery Corporation, Orlando, FL). Peracetic acid (35%) was obtained from FMC Corporation, Industrial Chemical Group, Princeton, NJ. A nonionic surfactant was used at a concentration of 0.05% (v/v) with chlorine, chlorine dioxide, Alcide, and peracetic acid.

Chemical determinations. Chlorine was measured as in previous studies (4) or with a test kit (model CLF-2B, CHEMetrics Inc., Calverton, VA). Chlorine demand of surface organic matter on unwashed fruit was determined by comparing the reduction in free residual chlorine caused by washing three unwashed or three previously washed fruit in 200 ml of chlorine solution (190 μg/ml, pH 7). Surface area of the fruit was measured to express the results in square centimeters of fruit surface. Methods to measure concentrations of chlorine dioxide were described previously (4). Water for hardness studies was prepared as described (2), and concentrations were measured with a kit (model PHT-CM-DR, LaMotte Chemical Products Company, Chestertown, MD).

RESULTS

In vitro comparisons. The isolate of *X. c.* pv. *vesicatoria* and the two isolates of *X. c.* pv. *citri* responded similarly to the various disinfectants in vitro. Exposures for 2 min were lethal with 1 μg/ml of chlorine or chlorine dioxide or a mixture of Alcide (1 sodium chlorite:80 water:1 lactic acid) diluted to a concentration of 6%. Quaternary ammonium or peracetic acid were lethal after 4 min at a concentration of 4 μg/ml. A concentration of 200 μg/ml of SOPP killed cells of the three bacterial isolates within 6 min.

Efficacy of disinfectants applied to unwashed fruit. Comparisons of the efficacy of the disinfectants applied as a spray for 1 or 2 min to unwashed fruit are shown in Table 1. Sprays for 2 min with chlorine or chlorine dioxide at 200 μg/ml significantly reduced the number of bacterial cells. However, some fruits sprayed with chlorine still supported survival counts exceeding 200 cfu. Sprays of quaternary ammonium (1,000 μg/ml) for only 1 min were equally effective to the chlorine compounds. SOPP (20,000 μg/ml) or peracetic acid (200 μg/ml) applied for 1 min were more effective than the 2-min sprays of chlorine or chlorine dioxide or the 1-min treatment of quaternary ammonium. The chlorine demand of unwashed fruit used in this study was 45.4 μg/cm of fruit surface, of which 41.0 μg was due to the surface organic matter.

When bacteria were suspended on membrane filters and subjected to the various disinfectants, all were lethal within 30 sec at the concentrations listed in Table 1.

Efficacy of disinfectants applied during washing. *Chlorine and chlorine dioxide.* Applications of chlorine or chlorine dioxide during washing reduced populations of *X. c.* pv. *vesicatoria* (Table 2). Chlorine dioxide at 100 μg/ml was as effective as chlorine at 150 or 200 μg/ml. A significant linear response of bacterial population to treatment concentration was only observed between recoveries from fruit surfaces and chlorine applications. Adjusting the pH of prepared solutions of chlorine dioxide, which were near pH 4, to a pH of 6 did not affect activity of the material.

Alcide, a chlorine dioxide-generating solution, was prepared by adding base (sodium chlorite) to water and then adding the activator (lactic acid). Preparations of 1:80:1 or 1:90:1 ratios were as effective as chlorine at 200 μg/ml. Higher dilutions did not reduce bacterial populations on fruit surfaces. Effective solutions of Alcide were near pH 3 and produced 1 μg/ml of chlorine dioxide upon mixing and a maximum of 4 μg/ml after 30 min. This preparation was effective if applied immediately or 30 min later. Adjustments of Alcide to pH 6 with potassium hydroxide immediately after preparation or after 30 min rendered the treatment ineffective. Dilutions of activator (lactic acid) with sterile tap water (1:80) exhibited eradicant activity in applications as 2-min sprays, as 30-sec washes, or in vitro on membrane filters. Aqueous dilutions of the base 1:80 were ineffective.

Quaternary ammonium compounds. Both formulations of the dual quaternary ammonium compounds were effective in wash applications for 30 sec (Table 3). A concentration of 500 μg/ml was as effective as approved levels of chlorine (Table 1). Populations of bacteria on treated fruit surfaces were significantly correlated with concentrations of Gallex and more effectively reduced with Bioserve at 500 or 1,000 μg/ml. Control with dual quaternary ammonium compounds was not affected by fruit rind temperatures of 8 or 27 C, nor did water hardness equivalent to 400 μg/ml of

Table 3. Reduction in populations of *Xanthomonas campestris* pv. *vesicatoria* applied to Valencia oranges and recovered after application of dual quaternary ammonium compounds during washing for 30 sec

Disinfectant (μg/ml)	Rating[y]					
	Gallex[z]			Bioserv		
	R	F	B	R	F	B
0	0.6 ± 0.2	1.8 ± 0.3	1.5 ± 0.2	...	...	...
500	0.1 ± 0.1	1.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.2	0.3 ± 0.3
1,000	0.1 ± 0.1	0.9 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
2,000	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1

[y] Ratings of 0, 1, 2, or 3 represent 0–5, 6–100, 101–200, and 201+ colony-forming units, respectively, per plate and are the means and standard errors of eight observations. Cells were recovered in the runoff (R) and from fruit (F) and brush (B) surfaces.

[z] Concentrations of Gallex were significantly ($P = 0.05$) correlated with recovery of bacteria from fruit surfaces ($r^2 = 0.944$).

calcium carbonate alter efficacy.

Peracetic acid. Treatments with peracetic acid during washing for 30 sec were very effective eradicants (Table 4) and were comparable to a spray treatment for 1 min (Table 1). An acceptable level of eradication comparable to approved chlorine treatments was achieved with peracetic acid at a concentration of 200 μg/ml. Solutions of peracetic acid at this concentration were near pH 4.0. Adjustment to pH 6.0 with potassium hydroxide did not affect bactericidal activity of applications used immediately after pH adjustment.

SOPP. Three formulations of SOPP were compared for efficacy in applications applied during washing for 30 sec (Table 5). All formulations effectively eradicated cells of *X. c.* pv. *vesicatoria* from the fruit surfaces. The SOPP soap and Dow-Hex formulations contained hexamine to reduce phytotoxicity, which may occur with a drop in pH below 11.5 (9). Lower concentrations of SOPP were not tested for disinfection, because a concentration at or near 2% is routinely used commercially for decay control (7) and lower concentrations would normally not be used.

Table 4. Reduction in populations of *Xanthomonas campestris* pv. *vesicatoria* applied to Valencia oranges and recovered after application of peracetic acid during washing for 30 sec

Peracetic acid (μg/ml)	Rating[z]		
	R	F	B
0	1.5 ± 0.3	2.3 ± 0.3	1.9 ± 0.3
200	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2
500	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0
1,000	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1

[z] Ratings of 0, 1, 2, or 3 represent 0–5, 6–100, 101–200, and 201+ colony-forming units, respectively, per plate and are the means and standard errors of eight observations. Cells were recovered in the runoff (R) and from fruit (F) and brush (B) surfaces.

Table 5. Reduction in populations of *Xanthomonas campestris* pv. *vesicatoria* applied to Valencia oranges and recovered after application of three formulations of sodium orthophenylphenate during washing for 30 sec

Disinfectant[x]	Rating[y]		
	R	F	B
Control	1.9 a[z]	2.1 a	2.1 a
Freshgard 5	0.3 b	0.3 b	0.6 b
SOPP soap	0.1 b	0.0 b	0.5 b
Dow-Hex	0.1 b	0.0 b	0.0 b

[x] All formulations were applied at a concentration of 20,000 μg/ml.

[y] Ratings of 0, 1, 2, or 3 represent 0–5, 6–100, 101–200, and 201+ colony-forming units, respectively, per plate and are the means of eight observations. Cells were recovered in the runoff (R) and from fruit (F) and brush (B) surfaces.

[z] Values within a column followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

DISCUSSION

On the basis of the similar response of the *X. campestris* pathovars to the various disinfectants in vitro, we conclude that these materials could be used effectively in the citrus canker eradication program for surface sanitation of asymptomatic fruit. Significant eradication of cells of *X. c.* pv. *vesicatoria* on surfaces of infested citrus fruit was achieved with all disinfectants evaluated. Applications during washing were effective within 30 sec of washing time. Concentrations (μg/ml) of the disinfectants, with efficacy comparable to or better than the presently approved treatments of chlorine or SOPP were chlorine dioxide (100), Alcide (1:80:1 dilution), dual quaternary ammonium compounds (500), and peracetic acid (200). Disinfectants other than SOPP or chlorine must be registered with FDA and EPA and approved for quarantine use by USDA/APHIS before they can be used in commercial channels.

Much of the applied chlorine was probably inactivated by the large amount of organic matter commonly present on Florida citrus fruit. This organic matter is composed mostly of mycelium of sooty mold fungi, dirt, and small amounts of epicuticular wax. Chlorine compounds are readily inactivated by organic matter (6), particularly hypochlorous acid, which is the more biocidal moiety. Survival of bacterial cells on unwashed fruit surfaces after chlorine treatments, even for 2 min, was probably due to inactivation by the organic matter. Chlorine dioxide is somewhat less reactive than chlorine and subsequently inactivated less readily (15). Results of this study confirmed this, showing that chlorine dioxide at one-half the concentration was as effective as chlorine. In wash applications, bacterial survival was often highest on fruit surfaces and could be correlated with treatment concentrations in the case of chlorine and Gallex. Once cells were dislodged from the fruit, disinfectants were more lethal, even at the lowest concentration. The bactericidal activity of Alcide apparently was due primarily to the lactic acid component because of inactivation at higher pH. Some effect also may have resulted from the slight amount of chlorine dioxide that was generated. Activity of peracetic acid was not pH-dependent. However, if use of solutions adjusted to pH 6 is delayed for several hours, efficacy could be expected to be reduced because of degradation of peracetic acid to acetic acid and oxygen at high pH (12).

Application of the disinfectants during washing would appear to have several advantages. The physical action of the brushes disrupts and removes surface organic matter and apparently improves the exposure of the bacteria to the toxicant. Continued application of the disinfectant during washing would replenish any that may have become inactivated. This would be particularly beneficial with a reactive material such as chlorine. Treatments applied during washing for 30 sec for bacterial eradication on asymptomatic fruit would not disrupt the orderly flow of fruit through the packing process, because this is the normal length of washing required for citrus under commercial conditions (8). Because SOPP is a proven decay control fungicide, it can be used for the dual purpose of decay control and bacterial eradication.

The approved treatments of chlorine for quarantine purposes could be reduced from 2 min to a 30-sec exposure if the disinfectant, when applied during washing, provided acceptable fruit cleanliness and did not cause excessive corrosion of the washing equipment. Likewise, soap or drench formulations of SOPP now requiring 45 sec and 1 min, respectively, could be applied within 30 sec to achieve eradicant activity.

ACKNOWLEDGMENTS

We thank Mark Davis, De-De Yawn, and Robert Leahy for technical assistance.

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