Bactericidal Treatment for the Eradication of *Erwinia amylovora* from the Surface of Mature Apple Fruit

W. J. JANISIEWICZ and T. VAN DER ZWET, Research Plant Pathologists, USDA-ARS, Appalachian Fruit Research Station, Kearneysville, WV 25430

ABSTRACT

Janisiewicz, W. J., and van der Zwet, T. 1988. Bactericidal treatment for the eradication of *Erwinia amylovora* from the surface of mature apple fruit. Plant Disease 72:715-718.

Several chemicals were evaluated for their effectiveness in eradicating viable *Erwinia amylovora* cells from aqueous suspensions and from the surfaces of artificially infested apple fruit. In vitro, 12 mg/L of sodium hypochlorite (NaOCl) and 62 mg/L of benzalkonium chloride (BC) eliminated all viable bacteria. Time of exposure to the bactericides had a significant effect on survival of *E. amylovora*. Addition of Ortho X-77 surfactant to the bactericides reduced the efficacy of the NaOCl solution, but had no apparent effect on BC. On artificially infested apple fruits, NaOCl at 450 and 500 mg/L plus 0.25% of surfactant greatly reduced, but did not totally eliminate, viable bacteria. All bacteria were eradicated from the apple surface with 1,400 mg/L of BC plus 0.5% surfactant. The eradicative effect was confirmed by passing buffer washings of treated fruit through millipore (0.2 μm) membrane filters that were subsequently incubated on selective media for possible development of *E. amylovora* colonies. Dip treatment with BC offers a possible remedy for meeting quarantine requirements for shipment of pome fruits to countries free of fire blight.

Additional keyword: *Malus*

Aside from the characteristic symptoms of fire blight (blossom, shoot, fruit, or trunk blight), the pear (*Pyrus communis* L.) or apple (*Malus* × *domestica* Borkh.) fruit surface may be infested with epiphytic *Erwinia amylovora* (Burr.) Winsl. et al. Isolation of such an epiphytic population has been reported from pear fruit in California (11) and apple fruit in West Virginia (20) and has been suspected as the cause of inadvertent dissemination to areas or countries free of fire blight (16,19). In Canada, however, Dueck (3) failed to detect epiphytic *E. amylovora* on mature cv. Wealthy apples collected from naturally infested orchards. The bacterium, applied as natural ooze or as a water suspension, did not survive for 24 hr on the surface of artificially infested fruit of cv. Delicious in the orchard, whereas survival was excellent on the surface of apples in the laboratory. When apples were infested with a water suspension of bacterial ooze (8.2×10^6 cfu/ml), a 10 min dip in 1.0 M of acetic acid was effective in reducing the population of *E. amylovora* (4).

Mention of a trademark, warranty, proprietary product, or vendor does not constitute a guarantee by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may be suitable.

Accepted for publication 21 March 1988.

Research has been under way at the Appalachian Fruit Research Station (AFRS) to develop methods of eliminating epiphytic populations of *E. amylovora* from the surface of fruit destined for export to countries free of fire blight. In 1985, van der Zwet and Walter (21) demonstrated that noninjured cv. Delicious fruit did not become infected when dip-inoculated for 10 min in a bacterial suspension (10^8 cfu/ml) of *E. amylovora*. In the laboratory, *E. amylovora* did not survive a 1-min exposure to 5% sodium hypochlorite (NaOCl), 0.1% acetic acid, or 50% ethanol (18). Solutions of NaOCl had been found to be an excellent disinfectant of pruning tools to eliminate *E. amylovora* (10). More recently, gamma radiation was found to totally eliminate *E. amylovora* from naturally infested fruit at dosages one third lower than the maximum rate tolerated by fruit if the bacterial populations were pretreated with a radiation-sensitizing chemical (8). The objective of this study was to find bactericides that could totally eliminate *E. amylovora* from the apple surface. A preliminary report has been published (7).

MATERIALS AND METHODS

Bacterium. *Erwinia amylovora* isolate Ea273 obtained from S. V. Beer (Cornell University, Ithaca, NY) was used for in vitro studies and for artificial infestation of fruit. The bacterium was grown in nutrient yeast-dextrose broth (NYDB) for 24 hr on a shaker (150 rpm/min) at 24 C. A working suspension of the bacterium was obtained by centrifugation of the liquid culture for 10 min at 7,000 rpm, resuspension of the pellet in sterile distilled water, and adjustment of the concentration based on optical density determined in a spectrophotometer. The bacterial suspension was used immediately following dilution of the stock suspension.

Bactericidal tests in vitro. Sodium hypochlorite (NaOCl), sodium orthophenylphenate (SOPP), benzoic acid, *p*-hydroxybenzoic acid, benzalkonium chloride (BC), and ascorbic acid were tested for bactericidal activity against *E. amylovora* during initial screening in vitro. The chemicals were tested at concentrations ranging up to 2,000 mg/L, except NaOCl, where the highest concentration tested was 200 mg/L. Those chemicals exhibiting bactericidal activity were also tested in mixtures with 0.25 and 0.5% v/v of a surfactant, Ortho X-77 (Chevron, Richmond, CA).

Aqueous solutions of NaOCl were prepared from commercial bleach containing 5.25% sodium hypochlorite. The total available chlorine concentration was determined with a phenylarsine oxide titration kit (Hach Company, Loveland, CO), with a minimum detection limit of 1 mg/L. Measurements were made immediately following preparations of the solutions and after 1 hr. To determine "chlorine demand" (2) caused by adding the surfactant to the hypochlorite solution, total available chlorine was determined for solutions used in vitro and for treatment of apples immediately following their preparation and after 1 hr. Also, the pH of freshly prepared NaOCl solutions, with and without the surfactant, was determined.

An aqueous suspension of *E. amylovora* was mixed with different concentrations of the test chemicals in plastic tissue culture plates to give a final concentration of 1×10^8 cfu/ml. The suspensions were mixed and 0.1-ml samples were removed at four different exposure times and plated directly on NYDA medium in plates. The plates were incubated for 48 hr at 25 C, and colonies were counted. After an additional 48-hr incubation, the plates were reexamined for possible appearance of new colonies. Each treatment was replicated three times.

To study the effects of the surfactant (Ortho X-77) on the bactericidal activity, tests were conducted as above, except that the surfactant was added to the

bactericides before mixing with the bacterial suspension. Each treatment was replicated three times.

Bactericidal tests on apples. Average size (7.5 cm diameter) apples of cultivar Rome Beauty, collected from an orchard not infected with *E. amylovora*, were used after approximately 2 mo of storage at 1 C. One day before artificial infestation with *E. amylovora*, the apples were surface-sterilized by spraying to runoff with 70% ethanol, and after 1 min were dried with tissue paper.

Apples were infested with *E. amylovora* strain Ea273 by 5-min dips in 200 ml of aqueous bacterial suspension (1×10^8 cfu/ml) contained in 600-ml beakers. Apples were periodically turned over to assure that all surfaces were in contact with the bacterial suspension. They were removed from the suspension and allowed to air dry for 1 hr. The infested apples were immersed in 200 ml of test solution contained in 600-ml beakers for 10 min at 23 C. Control solutions were water and water plus surfactant alone. The fruits were periodically turned to assure equal exposure to the chemical on all sides. Each treatment consisted of three single apple replicates.

After chemical treatment, apples were air-dried for 15–20 min, transferred to 600-ml beakers containing 200 ml 0.1 M phosphate buffer (5), agitated on a rotary shaker at 50 rpm for 10 min, and sonicated (Branasonic 527 Branason Co., Shelton, CT) for 30 sec. Samples (0.1 ml) of washings were plated directly onto Miller-Schroth (MS) selective medium (11). As an enrichment technique for detection of less than 2×10^3 cfu/fruit, 10 ml of the washings from each beaker were forced through millipore membrane filters (0.2 μm pore size), and the filters were incubated on MS medium. All plates were incubated for 48 hr at 24 C, and colonies were counted. The plates were reexamined after an additional 48-hr incubation.

Bactericidal suspensions in which artificially infested apples were dipped were also passed through millipore membrane filters. In this case, a volume of 200 ml was passed through the filter. The filters were treated as above. To determine detection limits of the procedure, experiments identical to the above (excluding the concentration of dip and wash solutions on filters) were conducted, except that three different concentrations of *E. amylovora* (8.9×10^3, 1×10^6, and 1.4×10^8 cfu/ml) were used for apple infestation, and NaOCl was not added to the dip solution.

RESULTS

Bactericidal tests in vitro. Of six chemicals tested for bactericidal activity against *E. amylovora*, only NaOCl and BC totally eradicated *E. amylovora*. Hypochlorite solutions were effective at 12 mg/L or higher concentrations at all four exposure times (Table 1). Benzalkonium chloride was effective at 62 mg/L only at 20- and 30-min exposure, at 125 mg/L at 10-min exposure and longer, and at 250 mg/L at all exposure times (Table 2).

Addition of the surfactant X-77 decreased the effectiveness of the hypochlorite solutions against suspended cells of *E. amylovora* (Table 1). The bacterial populations survived treatment with 50 mg/L of NaOCl for 5 or 10 min only when 0.5% X-77 was present. However, the surfactant had no effect on the efficiency of BC (Table 2).

The amount of total available chlorine in the solution decreased as the concentration of the surfactant increased. At the 0.25% concentration of the surfactant, "chlorine demand" for 6 and 12 mg/L of hypochlorite solution was 2 and 6 mg/L, respectively. Over the range from 25 to 100 mg/L, it was about 9 mg/L, and at the range from 200 to 500 mg/L, it was 5 mg/L. After 1 hr, "chlorine demand" increased additionally 1 mg/L for the two lowest concentrations of hypochlorite solution, about 4 mg/L for the concentration range of 25 to 100 mg/L, and 5 mg/L at the higher concentrations.

At the 0.5% concentration of the surfactant, "chlorine demand" for 6 and 12 mg/L of hypochlorite solution was 5 and 10 mg/L, respectively. Over the range from 25 to 250 mg/L, it averaged 14 mg/L, and at the range from 300 to 500 mg/L, from 8 to 10 mg/L, respectively. After 1 hr, no total available chlorine could be detected at the two lowest concentrations of hypochlorite solution. At the range from 25 to 500 mg/L, an additional increase in "chlorine demand" was from 5 mg/L at the lower to 1 mg/L at the higher hypochlorite concentration.

Table 1. Effect of surfactant and length of exposure time on effectiveness of sodium hypochlorite (NaOCl) on survival of *Erwinia amylovora* in vitro

Chemical treatment	Length of exposure (min)	Chemical concentration (mg/L) 0	6	12	25	50	100
NaOCl	5	+++[z]	+++	−	−	−	−
	10	+++	+++	−	−	−	−
	20	+++	+++	−	−	−	−
	30	+++	+++	−	−	−	−
NaOCl + X-77 (0.25%)	5	+++	+++	+++	+++	−	−
	10	+++	+++	+++	+	−	−
	20	+++	+++	+++	−	−	−
	30	+++	+++	+++	+	−	−
NaOCl + X-77 (0.5%)	5	+++	+++	+++	+++	+++	−
	10	+++	+++	+++	+++	+	−
	20	+++	+++	+++	+++	−	−
	30	+++	+++	+++	+++	−	−

[z] Symbols represent recovery of *E. amylovora* (average of three replicates): +++ = > 300 cfu/plate, ++ = 30–300 cfu/plate, + = 1–30 cfu/plate, − = 0 cfu/plate.

Table 2. Effect of surfactant and length of exposure on effectiveness of benzalkonium chloride on survival of *Erwinia amylovora* in vitro

Chemical treatment	Length of exposure (min)	Chemical concentration (mg/L) 0	62	125	250
Benzalkonium chloride	5	+++[z]	+++	+	−
	10	+++	+	−	−
	20	+++	−	−	−
	30	+++	−	−	−
Benzalkonium chloride + X-77 (0.25%)	5	+++	+++	+	−
	10	+++	+	−	−
	20	+++	−	−	−
	30	+++	−	−	−
Benzalkonium chloride + X-77 (0.5%)	5	+++	+++	+	−
	10	+++	+	−	−
	20	+++	−	−	−
	30	+++	−	−	−

[z] Symbols represent recovery of *E. amylovora* (average of three replicates): +++ = > 300 cfu/plate, ++ = 30–300 cfu/plate, + = 1–30 cfu/plate, − = 0 cfu/plate.

As the concentrations of NaOCl solutions increased, the pH of the solutions also increased. The greatest increase was at the lower concentrations (6–50 mg/L) of NaOCl. At these concentrations, the pH increased from 6.7 to 8.9, from 5.8 to 7.6, and from 5.5. to 7.1 in solutions without and with 0.25 and 0.5% of surfactant, respectively. At the higher concentrations, increases were smaller and at 500 mg/L of hypochlorite pH was 10.5, 10.0, and 9.3 for solutions without and with 0.25 and 0.5% of surfactant, respectively.

Bactericidal tests on apples. The surfactant had no significant effect on recovery of *E. amylovora* from apples infested with all three bacterial concentrations tested. However, on apples infested with the lowest concentration of the bacterium, tendency was observed toward lower recovery rate as the concentration of the surfactant increased.

Hypochlorite treatments at 450 and 500 mg/L plus 0.25% surfactant, led to wash solution recovery of 1 and 1.3 cfu per plate, the best treatments in the test (Table 3). These two treatments and 400 mg/L plus 0.25% X-77 were the only ones in which the filters contained colony numbers low enough to be counted.

Since there was no marked effect of the surfactant on BC effectiveness on apples tested in vitro, only BC plus surfactant at 0.25 and 0.5% was used (Table 4). At 1,000 and 1,200 mg/L of BC plus 0.25 and 0.5% of surfactants, respectively, live bacteria still occurred. The next higher concentrations of BC (2,000 mg/L and 1,400 mg/L) for those treatments, respectively, were effective in eliminating the bacterium. The absence of viable *E. amylovora* in phosphate buffer washings was observed both by direct plating as well as by placing filters with concentrated washings on the medium.

In both NaOCl and BC treatments in all concentrations tested, no bacteria appeared on filters through which the bactericidal solutions used for dipping infested apples were passed.

DISCUSSION

Differences between bactericidal effectiveness of NaOCl and BC in vitro and on apples were apparent. Treatment of infested apples with BC required about a 20-fold greater concentration than in vitro treatment to obtain total bacterial kill. In the case of NaOCl treatment, such an increase was not effective. Although addition of Ortho X-77 surfactant to NaOCl decreased the pH of the solution, suggesting enhanced bactericidal activity (2), at the same time it greatly increased "chlorine demand," resulting in lower effectiveness of the solution in vitro. Lower "chlorine demand" observed at higher concentrations of NaOCl solution may be a result of lower precision of the detection procedure at those concentrations. Tests on infested apples at 0.25% surfactant in the two highest NaOCl concentrations resulted in substantially greater mortality of bacteria than NaOCl alone or in mixture with higher concentrations of surfactant. Other than these two best treatments, large variations in NaOCl tests were observed that agree with others (12) who have used this compound for postharvest disease control. Better penetration of NaOCl plus surfactant was apparently responsible for the increase in effectiveness on infested apples. This penetration probably compensated for the "chlorine demand" resulting from addition of the surfactant. A similar effect was observed by Spotts et al (13–15), where improved control of fungal postharvest diseases of pears was

Table 3. Effect of sodium hypochlorite (NaOCl) plus a surfactant on survival of *Erwinia amylovora* on artificially infested apples, as determined by bacterial plate count from treated fruit washed and sonicated in buffer

Chemical treatment	Sodium hypochlorite concentration (mg/L) 0	300	350	400	450	500
Recovery of bacterium from fruit washings[w]						
NaOCl	+++[x]	++	++	++	+	++
NaOCl + X-77 (0.25%)	++	+++	+	++	+	+
NaOCl + X-77 (0.50%)	++	++	+	+	++	+
Recovery of bacterium from fruit washings through filter[y]						
NaOCl	+++	+++	+++	+++	+++	+++
NaOCl + X-77 (0.25%)	+++	+++	+++	++	++	++
NaOCl + X-77 (0.50%)	+++	+++	+++	+++	+++	+++
Recovery of bacterium from chemical treatment solution through filter[z]						
NaOCl	+++	–	–	–	–	–
NaOCl + X-77 (0.25%)	+++	–	–	–	–	–
NaOCl + X-77 (0.50%)	+++	–	–	–	–	–

[w] 0.1 ml of 200 ml of apple washings per plate.
[x] Symbols represent average of three replicates: +++ = >300 cfu/plate, ++ = 30–300 cfu/plate, + = 1–30 cfu/plate, – = 0 cfu/plate.
[y] Following concentration of 10 ml of washings on membrane filter.
[z] Following concentration of total volume of chemical treatment solution on membrane filter.

Table 4. Effect of benzalkonium chloride plus a surfactant on survival of *Erwinia amylovora* on artificially infested apples, as determined by bacterial plate count from treated fruit washed with buffer, following concentration of these washings and the chemical treatment solutions on filters

Chemical treatment	Benzalkonium chloride concentration (mg/L) 0	125	250	500	1,000	1,200	1,400
Recovery of bacterium from fruit washings[v]							
Benzalkonium chloride + X-77 (0.25%)	++[w]	++	+	+	+	*[x]	*
Benzalkonium chloride + X-77 (0.50%)	++	*	*	*	*	+	–
Recovery of bacterium from fruit washings through filter[y]							
Benzalkonium chloride + X-77 (0.25%)	+++	+++	+++	+++	+	*	*
Benzalkonium chloride + X-77 (0.50%)	+++	*	*	*	*	+	–
Recovery of bacterium from chemical treatment solution through filter[z]							
Benzalkonium chloride + X-77 (0.25%)	+++	–	–	–	–	*	*
Benzalkonium chloride + X-77 (0.50%)	+++	*	*	*	*	–	–

[v] 0.1 ml of 200 ml of apple washings per plate.
[w] Symbols represent average of three replicates: +++ = >300 cfu/plate, ++ = 30–300 cfu/plate, + = 1–30 cfu/plate, – = 0 cfu/plate.
[x] * = Treatment not tested.
[y] Following concentration of 10 ml of washings on membrane filter.
[z] Following concentration of total volume of chemical treatment solution on membrane filter.

attributed to better penetration of NaOCl into wounds after addition of surfactants Ortho X-77 or Ag-98. With *E. carotovora* (Jones) Bergey et al, the increased bactericidal activity of chlorinated water at lower pHs was associated with equal or lower effectiveness under natural application, when the solution was used for rot control on tomatoes (1). Better penetration of the solution with higher pH into infection courts was suggested as a possible explanation.

In our study, both NaOCl and BC killed all *E. amylovora* in dip solutions of these bactericides at all concentrations tested, since no colonies developed on filters through which those solutions were passed. However, NaOCl did not kill all bacteria on the fruit surface. This agrees with other observations (12,17), indicating that a large portion of the NaOCl action of reducing postharvest diseases of fruit and vegetables is by reducing microorganisms in wash water, but not the complete disinfestation of the produce surface. During the course of drying of the fruit after bactericidal treatment in our study, residual chlorine from hypochlorite dip apparently volatilized. However, the fate of residual BC was uncertain and possibilities exist of some carry-over to the wash solution.

Several factors could contribute to the lack of total effectiveness of NaOCl. One is the effect of organic particles that frequently make NaOCl ineffective (12). This was observed when phosphate buffer washings from treated apples were concentrated on filters, on which, following placement on the medium, bacterial colonies frequently developed from various organic particles. Another possible explanation could relate to recent findings by Hicks and Rowbury (6), who showed that *Escherichia coli* attached to the surfaces of glass beads were more resistant to damage and elimination by NaOCl in broth or water than in a free state. This effect could be explained in a number of ways, but the most probable is that attachment may involve few cell layers protecting the ones underneath, that attached bacteria could repair damage more efficiently, either because of higher nutrient levels or close proximity of the cells, or the close proximity of cells limits the amount of chlorine that contacts each cell.

The effectiveness of BC on apples at 1,400 mg/L and higher concentrations is very encouraging. The lack of growth of the bacterium on filters from apple washings in all effective concentrations from direct plating on the medium gave assurance that all bacteria had been killed. Following three months storage of apples treated with BC at 2,000 mg/L, no visual damage to apple skin was observed. Benzalkonium chloride, which is widely used in medicine as an antimicrobial preservative in pharmaceutical solutions, as an antiseptic for humans, and for equipment sterilization (9), may also be useful for postharvest treatment of fruit and vegetables for eradication of plant pathogenic bacteria.

ACKNOWLEDGMENTS

We wish to thank S. Taminovich, J. Walter, and D. Wydoski for their technical assistance.

LITERATURE CITED

1. Bartz, J. A. 1987. Effect of pH on efficacy of chlorinated water for control of postharvest disease in tomato fruit. (Abstr.) Phytopathology 77:1697.
2. Bartz, J. A., and Eckert, J. W. 1987. Bacterial diseases of vegetable crops after harvest. Pages 351-376 in: Postharvest Physiology of Vegetables. J. Weichmann, ed. Marcel Dekker, Inc., New York and Basel.
3. Dueck, J. 1974. Survival of *Erwinia amylovora* in association with mature apple fruit. Can. J. Plant Sci. 54:349-351.
4. Dueck, J. 1974. Bactericidal treatment of apples for elimination of surface-borne *Erwinia amylovora*. Can. J. Plant Sci. 54:353-358.
5. Goodman, R. N., and Shaffer, W. H. 1971. An inoculation procedure for evaluating the efficacy of toxicants against *Erwinia amylovora*. Pages 20-22 in: Proc. Workshop Fire Blight Res. 2nd. Michigan State University, East Lansing.
6. Hicks, S. J., and Rowbury, R. J. 1986. Virulence plasmid—associated adhesion of *Escherichia coli* and its significance for chlorine resistance. J. Appl. Bacteriol. 61:209-218.
7. Janisiewicz, W. J., and van der Zwet, T. 1987. Effect of two bactericides on *Erwinia amylovora* survival in vitro and in vivo on apples. (Abstr.) Phytopathology 77:987.
8. Janisiewicz, W. J., van der Zwet, T., and Jahrling, P. B. 1986. Laboratory studies on the effect of gamma radiation on survival of *Erwinia amylovora* on apple fruit. Can. J. Microbiol. 32:787-790.
9. Kaslow, R. A., Mackel, D. C., and Mallison, G. F. 1976. Nosocomial pseudbactermia. J. Am. Med. Assoc. 236:2407-2409.
10. Keil, H. L., and van der Zwet, T. 1967. Sodium hypochlorite as a disinfectant of pruning tools for fire blight control. Plant Dis. Rep. 51:753-755.
11. Miller, T. D., and Schroth, M. N. 1972. Monitoring the epiphytic population of *Erwinia amylovora* on pear with a selective medium. Phytopathology 62:1175-1182.
12. Smith, W. L. 1962. Chemical treatment to reduce postharvest spoilage of fruits and vegetables. Bot. Rev. 28:411-445.
13. Spotts, R. A., and Cervantes, L. A. 1986. Addition of the surfactant Ag98 to chlorine to improve control of decay of pear fruit. (Abstr.) Phytopathology 76:1065.
14. Spotts, R. A., and Cervantes, L. A. 1987. Effects of the nonionic surfactant Ag-98 on three decay fungi of Anjou pear. Plant Dis. 71:240-242.
15. Spotts, R. A., and Peters, B. B. 1982. Use of surfactants with chlorine to improve pear decay control. Plant Dis. 66:725-727.
16. Sprague, R., and Covey, R. P. 1969. Fungous and bacterial pear diseases of eastern Washington. Wash. Agric. Exp. Stn. Circ. 498.
17. Wilson, J. B., and Johnston, E. F. 1967. Reducing the incidence of bacterial lenticel infection on fall-washed Maine potatoes. Am. Potato J. 44:342.
18. van der Zwet, T. 1984. In vitro testing of various chemicals for bactericidal activity against *Erwinia amylovora*. (Abstr.) Phytopathology 74:825.
19. van der Zwet, T., and Keil, H. L. 1979. Fire blight—a bacterial disease of rosaceous plants. U.S. Dep. Agric. Handb. 510. 200 pp.
20. van der Zwet, T., and Van Buskirk, P. D. 1984. Detection of endophytic and epiphytic *Erwinia amylovora* in various pear and apple tissues. Acta Hortic. 151:69-77.
21. van der Zwet, T., and Walter, J. C. 1985. Significance of apple tissue injury to artificial infection by *Erwinia amylovora*. (Abstr.) Phytopathology 75:629.