Effects of Postharvest Chlorine and Wax Treatments on Surface Microflora of Lime Fruit in Relation to Citrus Bacteriosis Disease

J. J. STAPLETON, USDA, ARS, Apartado Postal 121, Tecoman, Colima, Mexico

ABSTRACT

Stapleton, J. J. 1986. Effects of postharvest chlorine and wax treatments on surface microflora of lime fruit in relation to citrus bacteriosis disease. Plant Disease 70:1046-1048.

Citrus bacteriosis (CB), a suspected form of citrus canker (*Xanthomonas campestris* pv. *citri* [*X. c.* pv. *citri*]), is expressed as lesions on leaves and twigs of Mexican lime (*Citrus aurantifolia*) as well as on other citrus plants in Colima, Mexico. Immersion of Mexican and Persian lime fruit in 200 ppm Cl, as sodium hypochlorite (NaOCl), for 2 min is a prerequisite for movement of fruit out of CB quarantine areas even though no bacteriosis symptoms have been observed on fruit. In addition, most Mexican citrus packers spray fruit with a protective wax coating before shipping. The effects of these treatments on lime surface microflora were evaluated. Total bacteria were reduced by 82–99+%, and fungi, by 81–100% in assays of fruit washings from limes treated with 50–900 ppm Cl as NaOCl. Nevertheless, total bacterial populations of 2.7×10^{2}–2.9×10^{3} cfu/cm^{2} of fruit surface survived Cl concentrations above the mandated 200-ppm level. No naturally occurring *Xanthomonas* spp. were recovered from fruit washings, although bacteria artificially inoculated in high concentrations were recovered at least 2 wk later on lime surfaces. Presumptive *X. c.* pv. *citri* was not eradicated when intact or wounded fruit were artificially inoculated with high concentrations of cells, then immersed in 200 ppm Cl for 2 min. The protective wax used in Colima did not increase the efficacy of Cl treatment.

In 1981, lesions of citrus bacteriosis (CB), a suspected form of citrus canker caused by *Xanthomonas campestris* pv. *citri* (*X. c.* pv. *citri*), were first observed on Mexican lime leaves and twigs in Colima, Mexico. Since then, similar symptoms were observed on Mexican lime trees in four other states (Jalisco, Michoacan, Guerrero, and Oaxaca) on the central Pacific coast of Mexico (7). Also, presumptive CB symptoms were observed on leaves and/or twigs of several citrus varieties other than Mexican lime in Colima (11). Bacteria identified as *X. campestris* based on physiological and biochemical tests, and provisionally as *X. c.* pv. *citri* based on pathogenicity, were occasionally isolated from CB lesions on leaves of Mexican lime. Characteristics of these isolates, however, differ from those isolated from other forms of citrus canker, so the involvement of other causal agents cannot be precluded (6,7). In an emergency action initiated to minimize the risk of CB spread, all lime fruit from quarantined areas in Mexico must be treated for 2 min in a sodium hypochlorite (NaOCl) solution containing 200 ppm Cl. This treatment was patterned after the one developed for export of Unshu orange to the United States from canker-affected areas in Japan (5); however, the effects of the treatment in Mexico were unknown. After NaOCl treatment, limes from Colima are usually sprayed with a wax preservative coating. This study was designed to determine the effects of the NaOCl and wax preservative treatments on organisms found on the surfaces of lime fruit in Colima, Mexico, and on a provisional *X. c.* pv. *citri* isolate from Colima.

Present address of author: Department of Plant Pathology, University of California, Davis 95616.

Accepted for publication 7 July 1986 (submitted for electronic processing).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

MATERIALS AND METHODS

NaOCl treatment of lime fruit. Dip-tank baths at three commercial Mexican lime packing sheds (containing 25, 110, and 500 ppm Cl) in Tecoman were assayed at random times for survival of bacteria and fungi by plating the NaOCl solutions on nutrient agar and potato-dextrose agar (PDA) (8). Plates were incubated at 28 C, and colony counts were made after a 4- to 7-day incubation period. Field-grown Mexican and Persian lime fruit were immersed in 50–900 ppm NaOCl for 2 min in randomly selected commercial packing sheds in Tecoman and/or in the laboratory. Three to five fruits were then transferred to sterile plastic bags containing 50–150 ml of phosphate-peptone buffer, pH 7.0. Bags were agitated for 30 min and the wash suspensions were dilution-plated on Wakimoto, nutrient, YDC, King's B, 523, and/or acidified PDA (3,8) to assay for reductions of bacteria and fungi. Fruit also were dipped in Cl (NaOCl) concentrations of 200, 400, 800, 1,200, and 1,800 ppm for 2 min, then waxed and incubated in paper bags at room temperature to assay for treatment toxicity to lime surfaces. Chlorine concentrations were determined iodometrically. The effect of the Cl treatment was evaluated by scanning electron microscopy (SEM) of fruit peel fixed by 3% glutaraldehyde immediately after treatment. Cl-treated and waxed fruit also were examined visually after 1- and 2-wk incubations.

Effect of wax preservative on *X. c.* pv. *citri*. The wax preservative used by most fruit packers in Colima (Pennwalt Citrus Lustr 266, containing alkali-soluble natural resins, silicon antifoam, and propylparaben [provided by Decco Tiltbed Division, Pennwalt Corp., Monrovia, CA]) was added in a logarithmic concentration series to nutrient agar and poured into culture plates. Cultures of the T20 isolate from Mexico provisionally identified as *X. c.* pv. *citri* (6,7,10) were streaked onto the plates, which were incubated and subsequently rated for bacterial growth 4 days later. In a second experiment, the Mexican isolate was grown in modified

Willbrink's medium broth (2) again augmented with a logarithmic concentration series of the wax product. After overnight growth, the shake cultures were spectrophotometrically assayed for bacterial growth. The effect of the wax spray treatment on fruit surface morphology was evaluated by observing peel sections fixed with 3% glutaraldehyde by SEM 2 and 48 hr after wax application.

Inoculation of fruit with presumptive *X. c.* pv. *citri* and treatment with NaOCl and/or wax. Intact or mechanically injured Mexican lime fruit were sprayed (hand-pump aerosol spray bottle) or swabbed to runoff with cotton containing either 10^4 or 10^9 cfu/ml of the (T20R^r) isolate. For ease in recovery and identification from fruit washings, the T20 strain was marked for resistance to 100 ppm rifampicin and judged identical to the wild-type strain. Inoculated fruit were incubated in ambient air or in plastic bags (to conserve humidity) and assayed for bacterial survival after incubation by plating fruit washings or macerates on YDC or nutrient agar with an amendment of 100 ppm rifampicin. Inoculated limes also were treated with 200 or 400 ppm Cl and/or wax and assayed for bacterial survival as described.

RESULTS

NaOCl treatment of lime fruit. Populations of live bacteria ranging from 1.7 to 52.0 cfu/ml were recovered from randomly sampled commercial packing shed dip-tank solutions. USDA import regulations require that dip-tank solutions be checked for [Cl] every 30 min, but concentrations tend to vary greatly. No fungi were recovered from any of the three Cl baths tested. All dip-tank solutions tested had pH values of about 8.4, which reportedly is near-optimal for Cl activity in commercial operations (1).

Total bacteria were reduced ($P<0.05$) in number by 77–99+% after dip-tank or in vitro NaOCl treatment (50–900 ppm Cl) of Mexican limes. These reductions were significant when both linear ($r = 0.77$) and quadratic ($r = 0.87$) regression factors were analyzed (Fig. 1). However, populations of 2.7×10^2–2.9×10^3 cfu/cm^2 of fruit surface remained at Cl levels above the mandated concentration of 200 ppm. No xanthomonad bacteria were recovered from either untreated or NaOCl-treated fruit. Total fungi were reduced by 81–100%, significant ($P<0.05$) for a curvilinear ($r = 0.82$) but not a linear relationship with increasing [Cl]. Similar reductions of microflora on Persian lime were found after in vitro NaOCl treatments (9). No apparent economic damage to fruit was observed after a 2-min treatment in NaOCl at Cl concentrations of up to 1,800 ppm followed by wax application. Damage that was observed visually, or by SEM, was very slight even 2 wk after treatment compared with untreated fruit, and probably would not be discernable to consumers.

Effect of wax preservative on bacteria. The wax product Citrus Lustr 266 had no effect on growth of the T20R^r strain in solid or liquid shake culture at concentrations of 10^4 ppm or less. Inhibition of bacterial growth did occur at 10^5 ppm. The scanning electron micrographs of Mexican lime fruit surface treated with wax showed a relatively heavy coating that became extensively cracked (perhaps caused by drying) after application compared with the natural fruit cuticle (Fig. 2). The cracking prevented complete sealing of the fruit surface by the wax layer.

Effects of NaOCl and/or wax on bacteria inoculated on fruit. Bacteria inoculated on fruit by aerosol spray at either concentration did not survive overnight incubation in open air or when inoculated fruit was completely dried before humid incubation. Inoculum swabbed on intact fruit at 10^4 cfu/ml did not survive overnight regardless of incubation method, but inoculum at 10^9 cfu/ml did survive a 2-wk incubation in plastic bags. Bacteria of both concentrations swabbed onto mechanically wounded fruit survived a 2-wk incubation in both plastic bags and open air. Bacteria were recovered from washings of intact and wounded fruit inoculated with 10^9 cfu/ml, then treated for 2 min with 200 or 400 ppm Cl with or without a subsequent wax coating. These treatments were done to test bacterial survival only; no effort was made to quantify results.

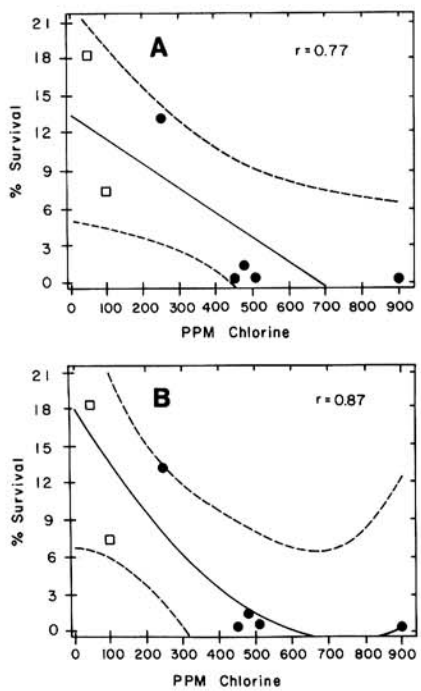

Fig. 1. Effect of chlorine concentration on survival of naturally occurring surface bacteria on Mexican lime fruit: **(A)** linear effect and **(B)** quadratic effect. □ = Chlorine treatment in laboratory and • = chlorine treatment in packing shed. Dashed lines indicate confidence limits at $P<0.05$.

DISCUSSION

Even though no fruit symptoms were observed on citrus from CB-infested areas (7,9), treatment of limes for 2 min in 200 ppm Cl (as NaOCl), which is used for exports from citrus canker-infested areas in Japan (5), was mandated by USDA as a prerequisite for exports of Mexican citrus into the United States. This study showed that some naturally occurring bacteria and fungi do survive NaOCl treatments on lime fruit surfaces in relatively high (10^2–10^3 cfu/cm^2) numbers. Although no naturally occurring xanthomonad bacteria were isolated

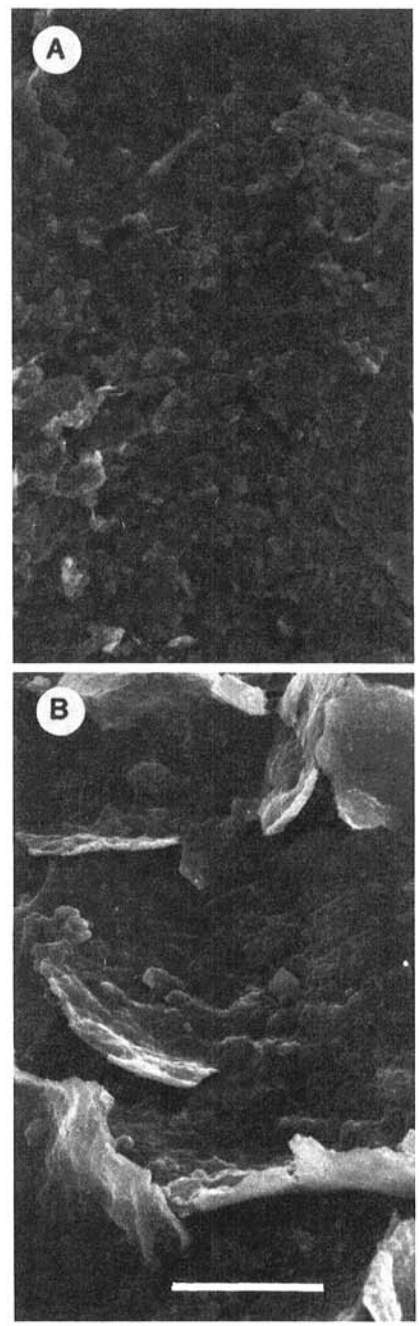

Fig. 2. Mexican lime fruit surface morphology: **(A)** natural and **(B)** coated with wax preservative. Scale bar = 20 μm.

from fruit during the study, their presence cannot be precluded, because the Mexican strain may be fastidious. This was the case with the Cancrosis B strain of *X. c.* pv. *citri* from Argentina (7). The failure of NaOCl to completely disinfest fruit allows the possibility of pathogen dissemination on Cl-treated fruit.

Results of a previous study showed that four strains of *X. c.* pv. *citri* did not survive a 2-min exposure to more than 10 ppm Cl (NaOCl) in aqueous suspension (10), suggesting that bacteria would not survive in packing shed dip-tank NaOCl solutions. However, both intact and wounded fruit surfaces may provide bacteria with protected sites, allowing them to avoid the effects of Cl treatment. In addition, fruit enter dip-tank solutions with some associated detritus and organic matter, which greatly reduces the bactericidal properties of Cl (4). The reduction, but not elimination, of microorganisms in dip-tank solutions and on plant surfaces treated by Cl in this report agrees with results of previous studies (1). The survival of bacteria on natural or artificially inoculated fruit treated at Cl concentrations several times the mandated 200 ppm, with or without a subsequent wax coating, indicated that although the NaOCl treatment is a relatively effective biocide, it is not an eradicant of fruit surface microflora. Because Cl concentrations as high as 900 ppm did not eradicate lime surface bacteria, increasing the mandated 200 ppm Cl concentration to improve disinfestation would not be effective.

The probability of disease spread from inoculum survival on fruit from Mexico may be very low, as suggested by the difficulty of infestation of the T20R^r isolate on fruit in this study as well as the lack of natural fruit symptoms in the field. However, if fruit treatments are desired for regulatory purposes to minimize the possibility of pathogen dissemination on fruit, the effects of disinfesting agents more active than NaOCl should be tested, both in dip-tanks and when added to protective wax formulations.

ACKNOWLEDGMENTS

I thank Noel Bello-Mayares and Pedro Rivera-Acevedo (USDA, APHIS, PPQ, LAR) for technical assistance in Mexico and Norita Chaney (USDA, ARS. Beltsville, MD) for electron micrographs. The assistance of USDA, APHIS, PPQ, LAR is appreciated.

LITERATURE CITED

1. Eckert, J. W., and Sommer, N. F. 1967. Control of diseases of fruits and vegetables by post-harvest treatments. Annu. Rev. Phytopathol. 5:391-432.
2. Koike, H. 1965. The aluminum-cap method for testing sugarcane varieties against leaf scald disease. Phytopathology 55:317-319.
3. Koizumi, M. 1971. A quantitative determination method for *Xanthomonas citri* by inoculation into detached citrus leaves. Bull. Hortic. Res. Stn. Jpn. (Ser. B.) 11:167-183.
4. McCulloch, E. C. 1936. Disinfection and Sterilization. Lea & Febiger, Philadelphia, PA. 525 pp.
5. Obata, T., Tsuboi, F., and Wakimoto, S. 1969. Studies on the detection of *Xanthomonas citri* by phage technique and the surface sterilization of Unshu orange for export to the United States. Res. Bull. Plant Prot. Jpn. 7:26-37.
6. Rodriguez G., S., Stapleton, J. J., and Civerolo, E. L. 1986. *Xanthomonas campestris* involved in Mexican lime bacteriosis in Colima, Mexico. In: Proc. Int. Conf. Plant Pathog. Bact., 6th. College Park, MD. In press.
7. Rodriguez G., S., Garza L., J. G., Stapleton, J. J., and Civerolo, E. L. 1985. Citrus bacteriosis in Mexico. Plant Dis. 69:808-810.
8. Schaad, N. W., ed. Laboratory Guide for Identification of Plant Pathogenic Bacteria. American Phytopathological Society, St. Paul, MN. 72 pp.
9. Stall, R. E., and Seymour, C. 1983. Canker, a threat to citrus in the Gulf-Coast states. Plant Dis. 67:581-585.
10. Stapleton, J. J. 1986. Effect of sodium hypochlorite on lime fruit surface microflora and suspensions of *Xanthomonas campestris* pv. *citri*, as related to citrus bacteriosis in Colima, Mexico. In: Proc. Int. Conf. Plant Pathog. Bact., 6th. College Park, MD. In press.
11. Stapleton, J. J., and Perez-Serrato, P. 1986. Observed host range and distribution of citrus bacteriosis symptoms in Colima, Mexico. (Abstr.) Phytopathology 76:376.