Susceptibility of *Corynebacterium sepedonicum* to Disinfectants In Vitro

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ABSTRACT

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Susceptibility of *Corynebacterium sepedonicum*, the causal bacterium of potato ring rot, to [illegible] disinfectants and heat was evaluated in vitro under varying conditions. The quantitative suspension test was used to measure survival of bacteria after 5 and 10 min exposure of aqueous suspensions with and without addition of organic matter, and for bacteria dried on wood carriers. The organism did not survive following most treatments, but there was more consistent control at 10 min exposure than at 5 min. The efficacy of hypochlorites and iodines was reduced by organic matter. Dried bacteria were generally as susceptible to the disinfectants as bacteria in suspension. Secondary inoculum of *C. sepedonicum* on infested surfaces and equipment can be effectively eliminated by most disinfectants provided the inoculum is in contact with the disinfectant for a minimum of 10 min. A minimum temperature of 87 C for 5 min was required for complete inactivation of bacteria. Other limitations, such as safety and corrosiveness, may affect the choice of disinfectant to be used.

Bacterial ring rot of potatoes, caused by the bacterium *Corynebacterium sepedonicum* (Spieck. & Kotth.) Skapt. & Burkh. (proposed synonym *Clavibacter michiganense* subsp. *sepedonicum* Davis et al [4]) has been in this country since 1939 and has caused sporadic but destructive outbreaks of bacterial ring rot of potatoes. Most disease outbreaks result from the use of infected seed potatoes because the bacterium is spread from diseased to healthy seed tubers during the cutting and planting process. Present control methods exclude primary inoculum by using clean stock programs combined with rigorous certification schemes centered on zero tolerance. These practices are not always effective because potatoes are vegetatively propagated and can become reinfected from secondary inoculum sources, or bacteria in a latent state can persist in seed lots between potato generations (15). Tests to detect latent bacteria, such as enzyme-linked immunosorbent assay (ELISA) or immunofluorescence (5), may not be reliable enough to preclude infected seed lots from being planted. Low numbers of undetected ring rot bacteria (30–100 colony-forming units) may cause disease and potential epidemics in subsequent generations of potatoes (15). For these reasons, it is imperative in ring rot-control strategies to eradicate all *C. sepedonicum*, not just reduce inoculum to low numbers.

The main secondary inoculum sources are potato production surfaces and equipment that become contaminated during contact with infected seed stocks. Contamination and infection of clean seed lots can occur if seed handling equipment is not properly sanitized. Although the bacterium does not produce spores, it can nevertheless persist in a dried state for up to 2 years on contaminated surfaces and longer in dried stems (14). The mechanism of persistence may be the viscous lipopolysaccharide capsule surrounding the cells that prevents desiccation. A commonly recommended control method for secondary inoculum is annual cleaning and disinfection of surfaces of potato production facilities and handling equipment using a disinfectant effective against the dried bacterial cells and slime (23). Although a large number of disinfectants have been recommended for this purpose, data are lacking as to the susceptibility of *C. sepedonicum* to many of the commonly recommended chemicals and treatment times. Most efficacy data for disinfectants have been generated from nonagricultural systems, such as the food industry and hospitals, using mammalian bacteria as test organisms.

There are relatively few reports on the susceptibility of plant pathogenic bacteria to disinfectants (9,13,20,24). The efficacy of disinfectants (1,6,7,10–12) or moist heat (17) for control of *C. sepedonicum* have been compared in only a few earlier reports. Despite limited research in this area, recommendations for disinfectants are confusing because of mixed or conflicting data and the fact that many compounds tested are no longer available or are prohibited for this use. In addition, most of the compounds were not tested under the dirty conditions often encountered in potato production, such as the presence of large amounts of organic material. Furthermore, most

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tests did not use dried deposits of bacteria, the predominant state in which *C. sepedonicum* persists on potato production facilities and equipment.

The purpose of this study was to test the effect of numerous disinfectants on the survival of *C. sepedonicum* under varying conditions including exposure time, bacterial state, and organic load. The intent was to determine reliable methods for control of secondary inoculum by approximating the conditions encountered in agriculture using *C. sepedonicum* as the test organism. This information is critical for preventing infection by, and spread of, *C. sepedonicum* among seed lots. The continued proper use of bactericidal disinfectants is a major factor in the national ring rot eradication campaign currently under way (8,22).

MATERIALS AND METHODS

Disinfectants tested. A total of 28 treatments including 15 proprietary compounds, five chemicals and heat were evaluated for their effectiveness in killing ring rot bacteria (Table 1). Seven of the treatments tested were hypochlorite-based, five were quaternary ammonium compounds, four were phenolics, three were iodines, and six were miscellaneous compounds, including Cu-8-quinolinolate, formaldehyde, acid mercury (2), 70% ethanol, hydrogen peroxide, and $CuSO_4$. Solutions of sodium hypochlorite were adjusted to pH 7.0 and diluted to 1:50, 1:100, 1:200, and 1:500 and one was tested at 1:50 without pH adjustment. Heat sensitivity was also tested by immersing wooden dowels infested with *C. sepedonicum* in water at temperatures of 49, 66, and 82 C. The bacteria were tested on the dowels in both a wet and a dried state. This test was conducted to simulate steam cleaning, which is often used to sanitize handling and storage equipment in the potato industry. The concentration of products used are listed in Table 1. For proprietary products, the highest concentration recommended by the manufacturer was used.

Bacterial cultures. Seven wild type strains of *C. sepedonicum* from our collection were used randomly throughout the study. Strains were isolated from potato tubers with bacterial ring rot and grown on yeast extract-dextrose-calcium carbonate medium (YDC) (21) that contains (g/L) dextrose, 15; yeast extract, 10; calcium carbonate, 5; and Bacto agar, 15. Identity of *C. sepedonicum* was confirmed by indirect fluorescent antibody staining using monoclonal antibodies (5), gram stain morphology, and colony growth characteristics (21). Five- to 8-day-old cultures collected from solid medium were used throughout the study.

Quantitative suspension test. The quantitative suspension test as described by Reybrouck (16) was used to measure the effect of disinfectants on survival of bacteria and was adapted for use under organic load and dried bacterial conditions. Briefly, the procedure is as follows. Bacterial suspensions were adjusted with sterile distilled water to A_{600} = 0 (approximately 10^8–10^9 cfu/ml), and 0.1 ml of the bacterial suspension was added to 10 ml of disinfectant at the test concentration. For the temperature treatments, the 0.1 ml of bacterial suspension was added to 10 ml of distilled

Table 1. Disinfectant treatments and rates tested to control of *Corynebacterium sepedonicum*

Disinfectant	Ingredients[a]/formulation	Source	Rate tested
Midland F-25	12% QAC[b]	Midland Laboratories, Dubuque, IA	1 oz/4 gal (2.0 ml/L)
San-O-Dis	10% QAC	Stein Chemical, Moorhead, MN	5 oz/5 gal (4.8 ml/L)
Roccal II	50% QAC, 5% ETOH	Hilton-Davis Chemical Co., Cincinnati, OH	1 oz/5 gal (1.6 ml/L)
Cetram CLA 20	20% QAC	Chelfis Inc., Midland, TX	1 oz/4 gal (2.0 ml/L)
Germ-O-Solv 2	4.5% QAC, 1.9% EDTA, 10% Na_2CO_3, 8.5% Na_2SiO_3	Rochester Midland Corp., Rochester, NY	2.5 oz/gal (19.8 ml/L)
Hilex bleach	5.25% NaOCl	Purex Corp., Lakewood, CA	1:50
Hilex bleach	5.25% NaOCl adjusted to pH 7.0	Purex Corp., Lakewood, CA	1:50
Hilex bleach	5.25% NaOCl adjusted to pH 7.0	Purex Corp., Lakewood, CA	1:100
Hilex bleach	5.25% NaOCl adjusted to pH 7.0	Purex Corp., Lakewood, CA	1:200
Hilex bleach	5.25% NaOCl adjusted to pH 7.0	Purex Corp., Lakewood, CA	1:500
Exspor	1.33% $NaClO_2$ + 15.8% organic acid, a.a. = ClO_2	Alcide Corp., Westport, CT	1:1:4 base:activator:H_2O
Rocodyne	19% povidone-iodine complex, 10% phosphoric acid	Rochester Midland, Rochester, NY	1 oz/gal (7.8 ml/L)
Wescodyne	17% povidone-iodine complex, 10% phosphoric acid	West Chemical Products Inc., Long Island City, NY	6 oz/5 gal (9.5 ml/L)
Betadyne	10% povidone-iodine complex, ETOH	Purdue Frederick Co., Norwalk, CT	Undiluted
Wescol	42% coal tar oils, 20% soap, 16% coal tar phenols, 10% phenyl phenols	West Chemical Products, Long Island City, NY	1:50
SL 500	5% benzyl-o-chlorophenol, 7% isopropanol, 5.9% soap, H_2O, EDTA	Lehn & Fink Division of Sterling Drug, Montvale, NJ	2 oz/gal (15.8 ml/L)
Mikrol 1.0	15% isopropanol, 7.5% K-ricinoleate, 5% o-phenylphenol, 2% benzylchlorophenol, 3% sec-amylphenol, 1.3% NaEDTA	Rochester Midland Corp., Rochester, NY	1 oz/gal (7.8 ml/L)
Lysol	16.2% soap, 2.8% o-phenylphenol, 2.7% benzylchlorophenol, 1.8% ETOH, 1.5% xylenol, 0.9 isopropyl alcohol, 0.7% NaEDTA	Lehn & Fink Division of Sterling Drug, Montvale, NJ	2.5 oz/gal (19.8 ml/L)
Penetrate	1% Cu-8-quinolinolate	Worlds Best Products, Inc., Lemon Mills, IN	Undiluted
Heat	49 C	—	
Heat	66 C	—	
Heat	82 C	—	
Formaldehyde	37% formaldehyde	—	0.37% (10 ml/L)
Acid mercury	0.12% $HgCl_2$	Mallinckrodt, Inc.	Undiluted
Ethanol	70% ETOH	—	Undiluted
Hydrogen peroxide	3% H_2O_2	Sigma	Undiluted
Copper sulfate	crystalline	Baker Chemical	25 g/L
Accord	2.27% $NaClO_2$ + 15.1% organic acid, a.a. = ClO_2	Grace Inc., Dallas, TX	1:1:10 base:activator:H_2O

[a] Liquid unless otherwise designated.

[b] QAC = Blend of alkyl dimethyl benzyl ammonium chlorides of varying C chain lengths (12–18). See Sheaver (13).

water that had been incubated in a tube by immersion in a water bath set at 44, 46, or 48 C. Following a 5 or 10 min exposure time, a 0.1 ml aliquot of the bacteria-disinfectant mixture was mixed with 0.9 ml of either distilled water alone or distilled water containing a disinfectant inactivator (18). The mixture was then serially diluted to 10^{-3} in sterile distilled water blanks. Samples (0.1 ml) of each of the four dilutions were plated on YDC medium and incubated at 28 C. Water treatments, in which distilled water replaced the disinfectant, were used as a positive control with each test. Both dilutions of each treatment were replicated three times.

The number of colony-forming units tabulated after 5–7 days of incubation was used to calculate a germicidal effect (GE) value (16) for each treatment using the mean of the three replications. To accommodate statistical accuracy and coincidental limitations, only colony counts between 30–300 plates were used to calculate GE values.

The GE value, a measure of the number of bacterial cells killed, were calculated using the following formula: $GE = \log N_0 - \log N_1$, where N_0 was the number of colony-forming units developed in the control series in which the disinfectant was replaced by distilled water, and N_1 was the number of colony-forming units counted after exposure to the disinfectant (16). The number of colony-forming units in the control plates varies in each experimental run, therefore the GE number varies from experimental run to experimental run. Because many runs were necessary to evaluate all chemicals for several replicates, it was necessary to put all GE numbers on an equal basis for direct comparison. This was done by comparing the GE of a treatment series to its own control for that experimental run, and converting to percent of survival. Percent of survival was calculated using the formula $1 - (\log N_0 - \log N_1 / \log N_0) \times 100$. Each chemical was tested three times and an average value was calculated. In our experiments $\log N_0$ ranged from 4.5 to 8.4, indicating the range of recoverable bacteria from initial inoculum to be from 3.2×10^4 to 2.5×10^8.

Susceptibility to each disinfectant treatment was determined under the following six conditions: aqueous suspensions of bacteria at 5 and 10 min exposure times, and with and without organic load.

Inactivators. Inactivators were used to neutralize disinfectants before dilution and subsequent plating to prevent an inhibitory carryover effect of disinfectant from being carried over to the recovery medium. Sodium thiosulfate (3.0%) was used as the inactivator for the hypochlorite, chlorine dioxide, iodine, and mercury based disinfectants (19). Dilution (no chemical) was used for the remaining disinfectants, since the neutralizers Tween 20, Tween 80, and lecithin (18) were toxic to *C. sepedonicum*.

Organic load. Not all disinfectant compounds work equally well in environments that are high in organic matter, such as the potato industry. Therefore, the germicidal exposure test was modified to simulate these conditions. During preparation of the test suspension of bacteria, organic load mixture was substituted for sterile water as the diluent. The mixture was composed of 5% bovine albumin (fraction 5) (16) and 5% yeast extract (3). The bacteria were incubated in this solution at room temperature for 5 min before exposure to the disinfectant.

Carrier tests with dried bacteria. Under actual conditions, disinfectants are used primarily against bacteria dried on production surfaces. It was, therefore, important in this study to test the susceptibility of *C. sepedonicum* under these conditions. Wood was chosen as the carrier for these tests because in practical testing wood was the most difficult to disinfect compared with paper, stone, burlap, steel, and plastic. Unfinished hardwood dowels, approximately 20 × 5 mm, were soaked in a suspension of *C. sepedonicum* at 1.0 in sterile distilled water for 30 min. The infested dowels were placed in uncovered sterile petri dishes and allowed to dry in a horizontal laminar flow hood. The dowels were dried for 1, 14, 35, or 168 days before disinfectant treatment in the first, second, or third tests, respectively. Following immersion of the dowels in tubes of disinfectant, the wooden dowels were placed in 0.9 ml of inactivator solution and thoroughly agitated to recover the bacteria.

RESULTS AND DISCUSSION

The control of *C. sepedonicum* in each of the disinfectant treatments varied depending on the disinfectant and conditions tested (Table 2). Many of the compounds tested effectively controlled *C. sepedonicum*, if used properly. Several treatments were not completely effective. The most critical factor affecting efficacy of the disinfectants tested was the treatment time. Sodium hypochlorite and two of the iodine, heat, and formaldehyde treatments were the least effective at 5 min treatment times. When the exposure time of the bacteria to the disinfectant was lengthened to 10 min, the effectiveness of these compounds

Table 2. Survival[a] of *Corynebacterium sepedonicum* after 5 and 10 min exposure to disinfectants under differing conditions

Disinfectant[b]	Chemical group	Bacterial suspension		Bacterial suspension + organic load[c]		Dried bacteria[d]	
		5 min	10 min	5 min	10 min	5 min	10 min
Midland 1-25	QAC[e]	38	>0	21	11	>0	69
San O Dis	QAC	11	>0	>0	27	0	>0
Roccal II	QAC	11	0	0	0	0	>0
Coman CTA-20	QAC	0	0	0	0	0	0
Clean-Quick 64	QAC	0	0	>0	0	0	>0
Hi Ice bleach	NaOCl	41	0	60	54	28	0
Hi Ice bleach	NaOCl	38	0	62	46	24	0
Hi Ice bleach	NaOCl	>0	0	42	33	47	>0
Hi Ice bleach	NaOCl	11	0	62	36	62	24
Hi Ice bleach	NaOCl	>0	0	47	>0	89	0
Exspor	ClO_2	11	0	0	0	0	0
Roccdyne	Iodine	63	0	89	>0	21	0
Wescodyne	Iodine	63	0	36	11	>0	11
Betadyne	Iodine	0	0	21	0	0	0
Weisol	Cresylic	71	0	0	0	0	0
V-1-200	Phenol	0	0	0	0	16	>0
Mineral 328	Phenol	11	0	0	0	22	0
Lysol	Phenol	11	0	22	0	0	0
Penetrate	Quaternary	21	0	0	0	0	41
Heat	Hot water	21	0	89	44	60	0
Heat	Hot water	32	0	53	81	35	0
Heat	Hot water	0	0	0	72	0	0
Formaldehyde	Aldehyde	99	60	66	96	61	0
Acra mercury	Mercury	11	0	0	0	0	0
Ethanol	Ethanol	11	0	46	0	0	0
Hydrogen peroxide	Peroxide	>0	0	>0	0	0	0
Copper sulfate	Cu sulfate	17	0	>0	0	0	0
Absolut	ClO_2	0	0	39	0	0	0

[a] Percent survival, calculated using $1 - [(\log N_0 - \log N_1)/\log N_0] \times 100$, where N_0 = count in the control units after exposure to water only, and N_1 = count after exposure to the disinfectant; mean of three experiments.

[b] See Table 1 for application formulations, active ingredients, and rate used.

[c] Organic load = 5% yeast extract plus 5% bovine albumin.

[d] Bacteria applied to wooden dowels and air-dried prior to treatment.

[e] QAC = Blend of alkyl dimethyl benzyl ammonium chlorides of varying C chain lengths (12–18).

[f] >0 indicates presence of <30 colonies.

was increased (Table 2). Exposure to a disinfectant for 10 min is consistent with our recommendations for off-season sanitation of potato production surfaces (23).

The addition of organic matter had a deleterious effect on some of the treatments, particularly hypochlorite at both 5 and 10 min exposure, and iodine compounds at 5 min (Table 3). These results are consistent with other data (18). Hot water treatments were also adversely affected by addition of organic matter. We found little difference in susceptibility of *C. sepedonicum* to the disinfectants between moist and dried deposits (Table 2).

Formaldehyde was found to be ineffective for all treatments, except for dried *C. sepedonicum* bacteria exposed for 10 min. It appears that formaldehyde is a slow-acting disinfectant, and that time of exposure was the most important factor affecting it. Based on the data presented here, coupled with potential environmental hazards, the use of formaldehyde as an industry standard for the sanitation of potato storages and equipment cannot be supported.

It appears, however, that most of the common disinfectants used by the potato industry are effective against *C. sepedonicum* if 10 min application or treatment times are used. It is imperative that any surface treated with an effective disinfectant be kept moist for the entire 10 min. However, some of the compounds tested have additional considerations important in their selection criteria. Coal tar compounds cannot be used because of potential carcinogenicity risks. A temperature of 82 C (180 F) for 5 min was necessary to ensure complete control of *C. sepedonicum*, but 44 C for 10 min was equally effective. Steam is often used improperly as a bacterial eradicant because during application it is easy to confuse condensed water vapor at temperatures of <82 C with steam. In addition, treatment times with steam for 5 or 10 min on any production surface would be difficult and time-consuming. Acid mercury, although extremely effective, is illegal to use because of residue problems. Mercury, copper sulfate, and hypochlorite solutions are corrosive to metal, and this must be taken into consideration when used. Copper sulfate and quinolinolate would probably be best used as wood treatments and may provide residual bactericidal protection. Surprisingly, hydrogen peroxide appears to be an effective disinfectant even though *C. sepedonicum* possesses the catalase enzyme. In subsequent in vitro testing, 50% solutions of isopropanol or methanol also killed *C. sepedonicum* (Secor, *unpublished*).

The data presented here clear up many misconceptions regarding the use and effectiveness of commercial disinfectants on the survival of *C. sepedonicum* for sanitation of infested surfaces as performed under commercial agricultural conditions. It is possible to provide firmer recommendations based on specific data. However, it is necessary to be sure the chemicals used for this purpose possess both federal and state registration for use as disinfectants in potato warehouses and on production equipment.

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