Control of *Xanthomonas campestris* pv. *campestris* in Crucifer Seed with Slurry Treatments of Calcium Hypochlorite

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ABSTRACT

Schultz, T., Gabrielson, R. L., and Olson, S. 1986. Control of *Xanthomonas campestris* pv. *campestris* in crucifer seed with slurry treatments of calcium hypochlorite. Plant Disease 70:1027-1030.

Sixteen treatments were tested as soaks or slurries to eradicate the cabbage black rot pathogen (*Xanthomonas campestris* pv. *campestris*) from *Brassica oleracea* seed. The pathogen was reduced to undetectable levels in one laboratory-infested lot and in eight of 15 naturally infected lots when seed were slurry-treated with calcium hypochlorite at 10–20 g a.i./kg of seed and left sealed in containers for 16 hr. Slurry treatments did not immediately reduce germination but did reduce germination of some lots 6 mo after treatment. Slurry treatment is more compatible than soaks with commercial seed-conditioning practices and has less potential for injury to seed or spread of nontarget pathogens.

Seed infected with *Xanthomonas campestris* pv. *campestris* is an important source of primary inoculum in black rot epidemics of crucifers (21). Western Washington has long been looked to as a source of pathogen-free seed. Washington State Department of Agriculture inspectors survey annually and have never reported black rot in western Washington crucifer seed fields (M. Long, Washington State Department of Agriculture, Yakima, *personal communication*). Because 80% of the U.S. and 30% of the world's cabbage seed is produced in Washington, it is important that this seed be pathogen-free. After the development of sensitive methods for detecting seedborne *X. c.* pv. *campestris* (8,12,14), the pathogen was reported for the first time on cabbage seed grown in western Washington in 1980 (11). H. S. Humaydan (Joseph Harris Seed Co., Rochester, NY, *unpublished*) has since found increasing numbers of *Brassica* seed lots grown in western Washington infected with *X. c.* pv. *campestris*. The standard recommendation for treating seed lots infected with *X. c.* pv. *campestris* is a hot-water soak at 50 C for about 30 min (2). This treatment is often reported to be detrimental to seed viability (2,15,20) and not always effective in controlling the pathogen (8,11,19). Chemical seed treatments tested more recently, including oxytetracycline, chlortetracycline, streptomycin (9), streptomycin-sodium hypochlorite (8), and hot copper acetate (13), though effective, have been phytotoxic.

We report results of tests conducted to find a safe eradicant seed treatment for *X. c.* pv. *campestris* in crucifer seed. A preliminary report of this work has been published (18).

Scientific Paper 7238. Project 0667. Washington State University, College of Agriculture Research Center, Pullman 99164.

Accepted for publication 1 July 1986 (submitted for electronic processing).

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MATERIALS AND METHODS

Laboratory-infested seed. A single lot of Early Jersey Wakefield cabbage seed was infested in the laboratory by slurry treatment with finely ground cabbage leaf residues infected with *X. c.* pv. *campestris*. Infested seed was air-dried and stored in a test tube in the refrigerator until used. This seed was used to screen various treatments for eradicant ability. Sixteen eradicant treatments were applied to this seed either as aqueous soaks from 30 min to 2 hr or as slurries kept in sealed containers from 10 min to 16 hr. A list of treatments and methods is detailed in Table 1.

Treated seed were dried on a laboratory bench for 1 or 2 days and assayed for *X. c.* pv. *campestris* by directly plating 100–200 seeds onto SX agar (16) and nutrient starch-cyclohexamide agar (NSCA [14]). Plates were incubated at 30 C for 10 days and observed for yellow (on NSCA) or purple (on SX) mucoid colonies around the seed. Suspected colonies were tested for pathogenicity as described later.

Naturally infected seed. Slurry treatments that eliminated *X. c.* pv. *campestris* from laboratory-infested seed and were not phytotoxic were tested further using seed that had been naturally infected under field conditions. Seed were slurry-treated with 10–20 g a.i. $Ca(OCl)_2$ per kilogram of seed and left sealed in a container for 16 hr. Both a modified direct plating (DP) method (12) and a modified liquid plating (LP) method (8) were used to determine treatment efficacy. Ten thousand seeds from each of 15 *B. oleracea* lots naturally infected with *X. c.* pv. *campestris* were used for each method. With the LP assay, seed were rinsed four times with 100 ml of sterile tap water to remove residual treatment material. Ten thousand seeds from each lot were placed in a flask with 100 ml of sterile water plus one drop of Tween 20 and gently shaken at room temperature for 4 hr. Washings were serially diluted and 0.1 ml plated in triplicate on SX agar, NSCA, NSCA with antibiotics (NSCAA [10]), and basal starch-cyclohexamide agar with antibiotics (BSCAA [10]). Plates were incubated at 28 C for 7 days and observed for colonies of *X. c.* pv. *campestris*. With the DP assay, 10,000 treated seeds were rinsed five times with sterile water and air-dried for 30 min. At this point, seed were plump but dry enough to plate using a vacuum seed-plating head. Two hundred seeds per plate were placed onto 15-cm-diameter plates of NSCA or SX agar, using a total of 50 plates per treatment. Before treatment, a single sample of 10,000 seeds from each lot was assayed using the LP method to confirm the presence of the pathogen. These plates were incubated at 28 C and observed for 14 days. Suspected colonies were tested for pathogenicity.

Greenhouse evaluation. Four thousand collard seeds naturally infected with *X. c.* pv. *campestris* were slurry-treated using 20 g a.i. $Ca(OCl)_2$ per kilogram of seed with just enough water to adequately cover the seed. After treating, seed were left in a sealed container for 16 hr. Treated seed were planted in washed sand in the greenhouse and bottom-heated to keep sand temperature at 20 C. Air temperature varied from 15 to 24 C. Seven thousand untreated seeds from the same lot were used as a control. Seedlings were observed for 4 wk for symptoms of black rot. Isolations were made from seedlings showing symptoms and tested for pathogenicity. This experiment was repeated later using 4,000 treated and untreated infected collard seeds and, as an added check, 4,000 untreated, uninfected cabbage seeds.

Phytotoxicity of treatments was evaluated by germinating seed using Association of Official Seed Analysts procedures (3). Seed treated with $Ca(OCl)_2$ also were tested for emergence in a greenhouse trial.

Pathogenicity testing. Pathogenicity was confirmed by inoculating young cabbage plants with cells derived from individual colonies grown on yeast extract-dextrose-calcium carbonate agar (YDC [22]) for 48 hr. Plants were inoculated by puncturing leaf petioles with a sterile 25-gauge needle covered with bacterial cells. After inoculation, plants were placed in a Percival growth chamber set at 28 C for 14 hr (days) and 20 C at night and 80% relative humidity. Plants were observed for 2–3 wk for black rot symptoms including black veins with yellow, wilted, or blackened leaf areas beyond the point of inoculation. Control plants punctured through leaf petioles with a sterile needle and treated as described never developed black rot symptoms.

Table 1. Percentage of seed with *Xanthomonas campestris* pv. *campestris* and germination after various treatments of artificially infested cabbage seed

Treatment[a]	Percent infested seed	Percent germination[b]
Allyl isothiocyanate liquid (Kodak) slurry		
1.3 g	8	...
2.5 g	10	...
5.0 g	10	...
10.0 g	1	...
Calcium hypochlorite (HTH 65G, Olin Inc.)		
20% (w/v) aqueous soak (30 min)	7	...
10-g slurry	2	...
20-g slurry	0	92
CGA-78039 50WP (Ciba Geigy) slurry		
0.2 g	83	...
0.3 g	64	...
0.6 g	9	...
1.3 g	2	...
2.5 g	1	...
Copper acetate soak		
0.5% (w/v) at 40 C for 20 min	0	5
Crushed cabbage seed slurry		
1 g crushed seed/1 g treated seed	2	...
Ethanol slurry (70 ml/kg)		
70% (w/v)	0	0
60% sealed 1 hr.	0	39
60% sealed 30 min	1	...
50%	2	...
Formaldehyde soak (40% liquid)		
1:128 (1 hr)	1	...
1:64 (1 hr)	0	88
Hot-water soak		
50 C for 30 min.	0	92
2-Hydroxypropyl methanethiosulfonate (HPMTS 80EC, Buckman Labs.) slurry		
0.6 g	1	...
1.3 g	0	96
2.5 g	1	...
3.1 g	...	90
5.0 g	0	73
10.0 g	0	0
KT-19827 (Jeersannidhi Anderson Inst.)		
20-cc slurry/sealed 20 min	100	...
Methyl isothiocyanate liquid (Nor-Am Inc.) slurry		
1.3 g	14	...
2.5 g	8	...
5.0 g	4	...
10.0 g	4	...
TCMTB, Nusan 30 Flowable (Wilbur-Ellis Co.)		
0.8-g slurry	5	...
Triphenyltin hydroxide 45.7WP (TH Agriculture and Nutrition) slurry		
0.3 g	7	...
0.6 g	63	...
1.3 g	30	...
2.5 g	2	...
5.0 g	9	...
10.0 g	2	...
20.0 g	1	...
Streptomycin, 500 ppm (MSD AGVET), 2-hr soak followed by:		
0.5% NaOCl shake for 30 min	0	66
2% $NaHPO_4$ rinse and NaOCl shake for 30 min	7	...
5% $Ca(OCl)_2$ shake for 30 min.	7	...
Zinc Omadine, 48% liquid (Olin Inc.)	31	...
10 g sealed 10 min	5	...
Tap water (1-hr shake)	5	...
Infested control	97	93
Uninfested control	0	94

[a] Rates are active ingredient per kilogram of seed unless otherwise noted. Slurry treatments were sealed for 16 hr unless otherwise noted. One to 200 seeds were treated, except 50 were used in methyl isothiocyanate treatments. All soaks were in aqueous solution.

[b] Germination of 200 seeds following Association of Seed Analysts wet blotter method, determined only for effective eradicant treatments and controls.

RESULTS

Treatments of laboratory-infested seed. Seven of the 16 basic treatments eliminated *X. c.* pv. *campestris* from artificially infested seed (Table 1). However, only $Ca(OCl)_2$, one HPMTS slurry treatment, a hot-water soak, and a formaldehyde soak did not substantially reduce germination (Table 1). Though effective and nonphytotoxic on artificially infested seed, the relative effectiveness and phytotoxicity of hot-water soaks has been investigated thoroughly and was not tested further (8,13,19).

Treatments of naturally infected seed. In 15 lots of naturally infected seed that were slurry-treated with $Ca(OCl)_2$, *X. c.* pv. *campestris* was detected from only four lots by LP assays (Table 2). Nine of the 11 treated lots that were negative for *X. c.* pv. *campestris* with the LP test were assayed further with the DP method. The DP assay recovered *X. c.* pv. *campestris* from three additional lots rated negative for *X. c.* pv. *campestris* after LP assay (Table 2). All untreated assays of each infected seed lot were positive. In one naturally infected lot of cauliflower seed, slurry treatments using 20 g a.i. $Ca(OCl)_2$ per kilogram of seed that were sealed for

1 hr or longer eradicated *X. c.* pv. *campestris*, whereas treatments sealed for 30 min did not. Further tests of effective slurry treatments using HPMTS proved phytotoxic on naturally infected seed lots and were discontinued.

In the first greenhouse trial using infected collard seed treated with $Ca(OCl)_2$, only one seedling showed symptoms of black rot. Of the untreated, infected seed planted as a control, 157 seedlings had symptoms of black rot. In a second greenhouse experiment using $Ca(OCl)_2$, no seedlings developed symptoms, whereas 26 seedlings in the untreated control had symptoms of black rot. None of the 4,000 uninfected cabbage seeds used as a control in the second greenhouse trial had any symptoms of black rot. Positive identification of black rot in seedlings showing symptoms was confirmed by pathogenicity testing.

Slurry treatments using $Ca(OCl)_2$ did not adversely affect germination or emergence of *Brassica* seedlings when seed were tested shortly after treatment (Table 3). However, some treated seed lots showed reduced germination after 8 mo of storage (Table 4). In addition, most stored lots took longer to germinate than untreated controls (Table 4).

Table 2. Recovery of *Xanthomonas campestris* pv. *campestris* from calcium hypochlorite slurry-treated and untreated, naturally infected *Brassica oleracea* seed after direct (DP) and liquid plating (LP) assays[a]

Seed	LP assay (cfu recovered)		DP assay (no. infected seed)[c]
	Treated[b]	Untreated	
Broccoli	0[c]	1.3×10^4	...
	6[c]	1.3×10^4	...
	35[c]	2.7×10^4	10[d]
Cabbage	0	2.0×10^2	...
	0	6.3×10^2	0
	1	3.7×10^2	...
Cauliflower	0	3.2×10^3	1
	0	1.2×10^2	0
	0	1.6×10^2	0
	0	3.0×10^2	0
	4	1.7×10^1	...
	0	3.7×10^2	1
	0	1.5×10^3	0
	0	2.7×10^3	0
Collards	0	2.2×10^3	2
Cabbage control	...	...	87[d]
Uninfected control	...	...	0[e,f]

[a] Based on 10,000 seeds unless otherwise indicated.
[b] Slurry-treated at 10 g a.i./kg unless otherwise noted. All slurry treatments sealed for 16 hr after treatment. Treated seed rinsed to remove residual $Ca(OCl)_2$ before assay.
[c] Slurry-treated at 20 g a.i./kg.
[d] Based on 1,000 untreated, infected seeds.
[e] Untreated.
[f] Based on 400 seeds.

DISCUSSION

Slurry treatments with $Ca(OCl)_2$ were effective in reducing levels of the pathogen and did not affect seed germination immediately after treatment (Table 3). However, the pathogen was not controlled in all naturally infected lots with this treatment. This is probably due to the location of the pathogen within the seed. Deeply seated bacteria in or under the seed coat may be protected from the bactericidal action of hypochlorous acid (1). It is interesting that slurry treatments of $Ca(OCl)_2$ were more effective than an aqueous soak of $Ca(OCl)_2$ (Table 1) and that longer sealing of slurry-treated seed (2 hr vs. 30 min) using $Ca(OCl)_2$ also was more effective. This may be due to chlorine gas having time to reach additional areas of the seed coat that harbor *X. c.* pv. *campestris*. No data are available on the depth of infection for those crucifer seed lots found to contain *X. c.* pv. *campestris*. Evidence presented by others (7,17) indicates that surface contamination of seed with dust containing pathogenic bacteria may be an important means of seed transmission of *Pseudomonas phaseolicola* and *X. campestris* pv. *malvacearum*. During threshing of *Brassica* seed crops, ample opportunity exists to contaminate the surface of uninfested seed with dust from dried, infected plant material. If crucifer seed are contaminated with infected residues in western Washington, it is likely that seed lots could be disinfected with slurry treatments of $Ca(OCl)_2$.

This treatment reduced the number of infected seed in most lots tested to a level that may not cause a high incidence of black rot under favorable field conditions (15). However, because of the limited quantities of naturally infected seed available, each assay consisted of single replication of 10,000 seeds. This is the minimum number of seeds recommended for routine testing of crucifer seed lots for *X. c.* pv. *campestris* (12). Because only one sample of 10,000 seeds was used, it is possible that low levels of the pathogen were present but escaped detection (6). Observing zero infected seeds in 10,000 using the DP assay means we are 95% confident that the true number of infected seeds is 0.03% or less.

No treatment currently exists that effectively eradicates the pathogen from all infected seed lots. Thus, seed lots found to contain this pathogen should be treated and reassayed to determine treatment effectiveness. Because of the time and labor required, the DP assay is not used in routine testing for *X. c.* pv. *campestris* even though it can be very sensitive in detecting infected seed (12). Our results indicate the DP method to be more sensitive than the LP method because *X. c.* pv. *campestris* was recovered in three additional lots that tested negative using the LP method. However, the direct plating method used in this experiment is useful for assaying highly contaminated lots to determine the effectiveness of seed treatments.

One infected seedling observed from 8,000 treated seeds tested in the greenhouse may have been a result of contamination resulting from both untreated, infected seed and treated seed being grown side by side on a greenhouse bench. Both the greenhouse evaluations and the DP assay demonstrated that slurry treatment with $Ca(OCl)_2$ can be effective in controlling *X. c.* pv. *campestris*.

Seed viability was only reduced when $Ca(OCl)_2$-treated seed were stored after treatment. No viability testing was done

Table 3. Germination[a] and emergence[b] of crucifer seed tested immediately after slurry treatment with calcium hypochlorite

Seed	Treated[c]	Untreated
Germination		
Broccoli	79	78
Cabbage	92	92
Cauliflower	96	97
Collards	98	94
Emergence		
Brussels sprouts	88	91
Cauliflower	75	74
Collards	98	98
Chinese cabbage	97	97
Chinese mustard	93	93
Kale	98	99
Mustard	95	98
Radish	99	98
Rutabaga	96	96
Turnip	98	98

[a] Germination based on the percentage of 200 seeds, using Association of Seed Analysts wet blotter method after slurry treatment with 20 g a.i./kg and being sealed 16 hr.
[b] Emergence based on the percentage of 200 seeds planted in the greenhouse after slurry treatment with 10 g a.i./kg and being sealed 16 hr.
[c] There is no significant difference between treated and untreated seed at $\alpha = 0.05$.

Table 4. Percent germination of *Brassica oleracea* seed 8 mo after slurry treatment with calcium hypochlorite

Seed	Germination[a]		Days to germinate	
	Treated[b]	Untreated	Treated	Untreated
Cabbage	87*[c]	93	11	4
	57*	70	11	7
	86*	95	11	7
	72*	87	11	4
	45*	57	11	7
	98	95	7	7
Cauliflower	98	95	12	5

[a] Percentage based on 200 seeds using Association of Seed Analysts wet blotter method.
[b] Slurry treatments used 10 g a.i./kg for cabbage and 20 g a.i./kg for cauliflower.
[c] * = Values between treated and untreated seed significantly different at $\alpha = 0.05$.

between 1 wk and 8 mo after seed were treated, and the point where viability begins decreasing after seed treatment was not determined. Viability of seed at the time of treatment may influence the reduction in viability over time. Lots that had a lower germination when treated showed a greater reduction in viability than those lots that had higher viability initially. At present, slurry treatment with $Ca(OCl)_2$ can be valuable to control *X. c.* pv. *campestris* in stock seed used to produce seed crops. Aqueous soaks using $Ca(OCl)_2$ have also been effective in controlling *X. c.* pv. *vesicatoria* on pepper seed without reducing germination (4). Slurry treatments reduce the potential for injury and spread of inoculum that is inherent with aqueous soaks and are more compatible with commercial seed-treating practices (5). If practical methods can be devised to remove or neutralize treatment residues before storage and thus improve long-term germination, $Ca(OCl)_2$ may provide a safe alternative to hot-water treatment for commercial seed lots infected with *X. c.* pv. *campestris*.

ACKNOWLEDGMENTS

This research was supported in part by grants from the American Seed Research Foundation, the Northwest Agricultural Research Foundation, and the Puget Sound Seed Growers Association. We also wish to thank Norman Schaad for supplying seed and assisting with this project.

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