Detection of *Xanthomonas campestris* pv. *carotae* in Carrot Seed

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ABSTRACT

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A method has been developed to isolate *Xanthomonas campestris* pv. *carotae* (*X. c. carotae*) in carrot seed lots. The method consists of a low-temperature (5 C) stationary aqueous soak of carrot seed in darkness for 18 hr followed by a vigorous shaking of the seed in water containing Tween 20, concentration of bacterial cells by centrifugation (7,796 *g* for 15 min), and plating of a dilution series of these cells onto a modified Kado and Heskett's D5 medium. The method yielded repeatable and consistent results in replicated testing of seed lots. The bacterium was detected in 34 of 35 seed samples from infected fields and zero of six seed samples from healthy fields. One naturally infected seed in 10,000 seeds can be detected by this method. This is the first report of direct isolation of *X. c. carotae* from carrot seed.

Bacterial blight of carrots, caused by *Xanthomonas campestris* (Pam.) Dows. pv. *carotae* Kendr. (*X. c. carotae*), was first reported in California in 1934 (9). The disease has since been reported in Idaho (2,10,17), Arizona, New Mexico (4), New York (13), Wisconsin (14), Australia (16), Canada (16), Italy (3), and Japan (12). It is of increasing concern in carrot production fields in Florida (J. O. Strandberg, *personal communication*). Seed transmission of the disease has been demonstrated by Kendrick (9) with artificially infested seed and by Ark and Gardner (1) with naturally infected seed. Several investigators (1,2,9,10,17) have observed bacterial blight in carrot seed umbels. An important means of control will be the use of clean carrot seed. Effective eradicant seed treatments and control procedures in carrot seed fields are vital to the production and availability of clean carrot seed.

An accurate method is needed to detect the pathogen in and on seed, to evaluate disease control programs in seed fields, to check the efficacy of seed treatments, to conduct epidemiological studies, and to establish inoculum thresholds resulting in seed transmission. This paper expands on a preliminary report (11) of this method.

MATERIALS AND METHODS

Seed sources. Forty-one seed lots collected from healthy or naturally infected seed fields were used to test this method. Tests of several seed lots were replicated at different times to confirm the repeatability of the test method.

Isolation media. Kado and Heskett's (8) D5 medium was modified (MD5) by substituting filter-sterilization (0.22 μm) for heat-sterilization of D (+) cellobiose (Sigma Chemical Company, St. Louis, MO) and by addition of cycloheximide (final concentration 200 mg/L) (Actidione; Upjohn Company, Kalamazoo, MI). These were added after the autoclaved basal medium cooled to 60 C. Media-filled plates were dried for 3 days at room temperature to eliminate surface moisture before use. Recovery of bacteria on MD5 medium was compared by dilution plating with recovery on yeast-extract nutrient agar (YNA) (7) using three isolates of *X. c. carotae* obtained from J. O. Strandberg (Agricultural Research and Extension Center, P.O. Box 909, Sanford, FL) and seven pathogenic isolates obtained from plant samples.

Extraction and isolation of *X. c. carotae* from carrot seed. In the basic seed-soak extraction method, 20-g seed samples (about 10,000 seeds) were added to 100 ml of sterile cold aqueous 0.85% NaCl (saline) in 250-ml Erlenmeyer flasks. The flasks were swirled by hand to submerge the seed and incubated in the dark for 16–18 hr at 5 C. After incubation, 0.05 ml of autoclaved Tween 20 (J. T. Baker Chemical Company, Phillipsburg, NJ) was added to the flasks and the samples were shaken vigorously for 1 min. Suspensions were filtered through sterile cheesecloth. The retained seed were rinsed further with 25 ml of cold sterile saline. The pooled washings were collected in a clean sterile flask and centrifuged at 7,796 *g* for 15 min. Pellets were resuspended in 10 ml of sterile cold saline and the suspension was then diluted 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}. One-tenth of a milliliter of each dilution was plated in triplicate onto MD5 agar medium.

Seed rinsing was compared with seed soaking as a means of extracting the bacteria. Twenty grams of carrot seed were shaken vigorously in 100 ml of cold sterile saline for 30 sec; the suspension was then filtered through sterile cheesecloth and collected in another sterile flask. This was then centrifuged. The pellet was resuspended, diluted, and aliquots plated as described before. To provide comparison seed-soak data, 100 ml of fresh sterile saline was poured onto the rinsed seeds, extracted, and plated as before.

Evaluation and identification of *X. c. carotae*. The pathogen and saprophytic bacteria were evaluated after inoculated plates were incubated in the dark at 30 C for 7–10 days. On the MD5 medium, colonies of *X. c. carotae* were typically straw yellow, glistening, round, smooth, convex with entire margins, and 3–5 mm in diameter. Colonies typical of *X. c. carotae* were purified by two successive streak transfers from single colonies to plates of YNA before further testing.

All suspected isolates of *X. c. carotae* were tested for pathogenicity on seedlings of *Daucus carota* L. var. *sativus* Hoffm. 'Chancellor' at the second- to fourth-true-leaf stage by atomizing the leaves to runoff with bacterial suspensions of 1×10^6 colony-forming units (cfu) per milliliter. At least five plants were inoculated with each bacterial isolate tested. The inoculated plants were incubated at 100% RH and 25–30 C in a dew chamber (Percival Manufacturing Company, Boone, IA) for 48 hr to allow infection. After incubation, plants were set in a cool (20 C) greenhouse headhouse for 16 hr to minimize environmental shock before the plants were placed in the greenhouse at 19–32 C. Plants were watered from below to prevent splash contamination of the pathogen. Lesions appeared 7–10 days after inoculation. Plants atomized with water served as controls.

In addition to pathogenicity tests, isolates obtained from seed were tested biochemically for proteolysis of milk (casein hydrolysis), anaerobic growth, urease production, catalase production, aesculin hydrolysis, starch hydrolysis, production of fluorescent pigment on King's medium B, and arginine dihydrolase activity (6,7). Ten known pathogenic isolates were included as controls for all

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biochemical and pathogenicity tests.

Sensitivity of test method. Seed lots with various levels of infection were produced by adding 1, 2, 10, or 100 artificially infested or naturally infected seeds to 20-g samples of carrot seed from lots previously tested and found negative for *X. c. carotae*. Artificially infested carrot seed were prepared by incubating 1 g of seed in saline suspensions of *X. c. carotae* for 15 min in the dark at 30 C and then air-drying the seeds at room temperature for 24 hr. Different levels of infestation were also obtained by varying the concentration of *X. c. carotae* in the inoculum suspensions. The approximate number of colony-forming units after artificial inoculation was determined by individually soaking three inoculated seeds and isolating as previously described. Naturally infected seed were collected from severely infected umbels and tested with the quick-rinse method for the presence of *X. c. carotae*. The approximate concentration of inoculum per seed in naturally infected seed was determined by soaking 10 individual seeds and plating dilutions of the suspensions onto MD5 medium.

Seed transmission. Four seed samples were used to test seed transmission. Two lots were naturally infected. One lot was tested and found to be apparently free of pathogenic bacteria. The fourth lot was artificially infested with about 1×10^6 cfu per seed. In one trial, 100 seeds from each lot were planted singly in test tubes containing water agar. These tubes were incubated in a growth chamber at 30 C with a 12-hr/day light cycle. Fourteen days after planting, cotyledons showing symptoms were counted. In another trial, three replicates of 400 seeds from each of the four seed lots were planted in steam-pasteurized U.C. soil mix and placed in the greenhouse at 19–32 C. Pots were separated and watered from below to prevent splash distribution of the pathogen. Thirty days after planting, germinated seedlings and true leaf infections were counted. Pathogen identity was confirmed in both trials by isolation and pathogenicity tests.

RESULTS

Effectiveness of test method. When naturally infected seed were tested by seed soaking, we failed to isolate *X. c. carotae* on Kado and Heskett's D5 medium. However, we were successful when filter-sterilized cellobiose was substituted and cyclohexamide added. *X. c. carotae* was detected nine of 10 times when single seed from infected umbels were tested separately using this modification of D5 media (MD5). The concentration of *X. c. carotae* per seed ranged from 1×10^2 to 1.8×10^5. The recovery rate of 10 isolates of *X. c. carotae* on MD5 compared with YNA was 0.86 (range 0.82–0.93).

The concentration of *X. c. carotae* isolated from naturally infected seed by seed rinsing was consistently lower than by seed soaking (Table 1). In two cases, *X. c. carotae* was not isolated by the seed-rinsing method but was isolated by the seed-soaking method.

The pathogen was detected with the soaking method in 34 of 35 seed samples harvested from infected fields. It was not detected in six seed samples from healthy fields. When seed samples were retested, consistent results were obtained (Table 2). In another experiment, the method differentiated six infected seed samples treated with experimental eradicants from untreated samples in a blind test.

All isolates of *X. c. carotae* recovered from seed assays were pathogenic on carrot. All isolates were positive for proteolysis of milk (casein hydrolysis), aesculin hydrolysis, and catalase production. They were negative for anaerobic growth, urease production, starch hydrolysis, production of fluorescent pigment on King's medium B, and arginine dihydrolase activity. These reactions are typical for *X. c. carotae*.

Sensitivity of test method. The pathogen was consistently isolated from seed samples at various infection levels produced by the addition of artificially inoculated seed down to one infested seed with about 1.2×10^3 cfu in 10,000 clean seeds (Table 3). When infested seed contained 1.1×10^2 cfu, *X. c. carotae* was detected in only one of three replicates. The number of pathogenic bacteria isolated from seed samples increased when either the infested seed contained more bacteria or when more artificially infested seed were added to the samples.

The pathogen was consistently detected

Table 1. *Xanthomonas campestris* pv. *carotae* (*X. c. carotae*) and saprophytes detected on modified D5 media in naturally infected carrot seed lots with rinsing or soaking methods

Seed sample no.	Extraction method[a]	*X. c. carotae*[b] (cfu)	Saprophytes[c] (cfu)
1	Rinsing	4.8×10^6	2.2×10^8
	Soaking	1.5×10^8	2.6×10^8
2	Rinsing	1.0×10^3	1.2×10^6
	Soaking	8.8×10^6	1.9×10^8
3	Rinsing	0	5.9×10^4
	Soaking	4.0×10^4	1.6×10^8
4	Rinsing	0	3.1×10^4
	Soaking	1.6×10^6	5.7×10^6

[a] Rinsing: 20 g of carrot seed was vigorously rinsed with 100 ml of cold sterile saline for 30 sec. The saline was filtered through cheesecloth and centrifuged at 7,796 *g* for 15 min. The pellet was resuspended in saline, diluted, and plated on modified D5 medium. Soaking: the same seed, after rinsing, were soaked in 100 ml of cold sterile saline at 5 C for 16–18 hr. Filtration, centrifugation, and dilution plating were the same as with seed rinsing.

[b] Number of colony-forming units of *X. c. carotae* isolated on modified D5 medium from 20-g samples of carrot seed (about 10,000 seeds). Identification was confirmed by pathogenicity on seedlings of *Daucus carota* var. *sativa* 'Chancellor.'

[c] Number of colony-forming units of bacterial saprophytes isolated on modified D5 medium.

Table 2. *Xanthomonas campestris* pv. *carotae* (*X. c. carotae*) in seed lots from naturally infected and healthy carrot seed fields evaluated in different tests on modified D5 medium[a]

Seed sample no.	Seed field disease rating	Test	*X. c. carotae*[b] (cfu)	Pathogenicity[c]	Saprophytes[d] (cfu)
5	Diseased	1	1.9×10^6	+	7.8×10^6
		2	5.6×10^5	+	9.3×10^7
		3	6.0×10^5	+	2.4×10^6
6	Diseased	1	5.6×10^5	+	3.8×10^7
		2	1.6×10^6	+	4.2×10^6
		3	1.6×10^6	+	9.3×10^6
7	Healthy	1	0	NA	9.6×10^5
		2	0	NA	1.1×10^7
		3	0	NA	8.5×10^5

[a] Kado and Heskett's D5 medium (6) was modified by adding cycloheximide (200 mg/L) and filter-sterilized cellobiose after autoclaving the basal medium ingredients. Bacteria were extracted by soaking.

[b] Number of colony-forming units of *X. c. carotae* isolated from 20-g samples of carrot seed (about 10,00 seeds).

[c] Determined by spray inoculation of *Daucus carota* var. *sativus* 'Chancellor': NA = not applicable.

[d] Number of colony-forming units of saprophytic bacteria isolated from 20-g samples of carrot seed (about 10,000 seeds).

Table 3. *Xanthomonas campestris* pv. *carotae* (*X. c. carotae*) recovered from seed lots infected by adding artificially inoculated seed to clean seed

No. of infested seeds/10,000	*X. c. carotae* added[a] (cfu)	*X. c. carotae* recovered[b] (cfu)
100	4.6×10^8	$4.2–6.8 \times 10^7$
10	4.6×10^7	$4.7–10.0 \times 10^6$
2	9.2×10^6	$2.2–3.8 \times 10^6$
1	1.2×10^5	$7.8–20.0 \times 10^4$
1	1.5×10^4	$2.4–20.0 \times 10^4$
1	1.2×10^3	$1.2–3.2 \times 10^3$
1	1.1×10^2	$0–3.6 \times 10^2$
0	0	0

[a] Determined by replicate soaking of three individually infected seeds from each artificially infected lot.
[b] Range of colony-forming units of *X. c. carotae* recovered in three tests.

Table 4. *Xanthomonas campestris* pv. *carotae* (*X. c. carotae*) isolated from seed lots produced by adding naturally infected seed to clean seed

No. of infected seeds/10,000	*X. c. carotae*[a] (cfu)	Saprophytes[a] (cfu)
100	$3.2–4.3 \times 10^5$	$1.8–1.9 \times 10^6$
10	$2.1–3.9 \times 10^4$	$2.8–7.3 \times 10^5$
2	$0.85–7.8 \times 10^3$	$0.12–2.3 \times 10^3$
1	$2.8–8.0 \times 10^2$	$0.95–15 \times 10^4$
0	0	$1.9–5.3 \times 10^4$

[a] Range of colony-forming units of *X. c. carotae* and bacterial saprophytes isolated in three tests.

when only one naturally infected seed was added to 10,000 clean seeds (Table 4). Recovery of *X. c. carotae* increased as more naturally infected seeds were added to the samples.

Seed transmission. Seed transmission was demonstrated in tests on water agar and in steam-pasteurilized soil. In test tubes on sterile water agar, 31.4% of the cotyledons from artificially inoculated seed and 4.3% of the cotyledons from naturally infected seed developed symptoms. No symptoms were found on cotyledons from healthy seeds. In a greenhouse trial, germination of naturally infected, artificially infested, and clean seed ranged from 68 to 92%. Seed transmission averaged 0.6 and 0.9% of the emerged seedlings in the naturally infected lots, 2.7% in the artificially infested lot, and 0% in the clean lot.

Seedlings from clean seed did not develop symptoms. Pathogenic *X. c. carotae* was recovered from cotyledon and leaf infection in both tests.

DISCUSSION

This is the first report of direct isolation of *X. c. carotae* from carrot seed. It also confirms the reports of Kendrick (9) and Ark and Gardner (1) of seed transmission carrot bacterial blight.

An assay for seedborne bacterial pathogens requires two steps: extraction of the pathogen from seed and differentiation from associated seed saprophytes. The success of this test is due to both the modification of the D5 medium and an efficient bacterial extraction method. Prolonged soaking of seed (16–18 hr) enhanced extraction of *X. c. carotae* over rinsing, and the low temperature (5 C) during soaking apparently slowed multiplication of bacterial saprophytes and allowed *X. c. carotae* to be identified later on MD5 medium. Some of bacterial saprophyte colonies were extremely small (pinpoint). This allowed detection of *X. c. carotae* in the presence of high levels of saprophytes (Table 1, sample 3). Since the number of colony-forming units of *X. c. carotae* recovered was not higher than the number that had been added (in most cases) (Table 3), it is reasonable to assume that bacterial saprophyte multiplication was also slowed during the cold-soak period.

The function of the plating medium was to allow differentiation of *X. c. carotae* from seed saprophytes, not necessarily total inhibition of saprophytes. The D5 medium has been reported to be selective for *Xanthomonas* and *Agrobacterium* spp. (8) and has been used to isolate *Xanthomonas* spp. from plant (5) and seed samples (15). In our experience, filter sterilization of cellobiose to support growth of *X. c. carotae* and addition of cycloheximide to inhibit fungal saprophytes were necessary to isolate *X. c. carotae* successfully from carrot seed. With these modifications, 1×10^2 cfu of *X. c. carotae* can be detected in 10,000-seed samples. The method has been used to evaluate the effect of antibiotic applications to seed fields and the effectiveness of an experimental eradicant seed treatment (T.-L. Kuan and R. L. Gabrielson, *unpublished*).

Although other nonpathogenic yellow bacteria grew on MD5, with experience, these could be distinguished from *X. c. carotae* by colony characteristics alone. In addition, casein hydrolysis is an outstanding biochemical characteristic of *Xanthomonas* spp. (6) and can be used to identify the bacterium within 2 days compared with 2–3 wk for isolation and pathogenicity tests. Casein hydrolysis could thus speed evaluation of the effectiveness of seed treatments, field disease control programs, and epidemiological studies.

It should be emphasized that pathogenicity tests should always be conducted for regulatory purposes since pathogenicity is of primary concern. Correlation between levels of seed infection and subsequent development of disease in the field causing monetary loss remain to be determined. This will require data from field trials under environmental conditions favorable for infection using seed lots with different pathogen levels. Without this, the method should not be used for routine regulatory purposes.

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