A Qualitative Method for Detecting Xanthomonas campestris in Crucifer Seed

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ABSTRACT

An assay procedure using water-enriched cabbage seeds on plating media containing cycloheximide was developed for detecting low numbers of *Xanthomonas campestris,* the causal agent of black rot of crucifers.

Using inoculated seed, this method was capable of detecting a single diseased seed per 1,000 and was superior to several other methods tested. A commercial lot of cauliflower seed was shown to contain 0.8% diseased seed. A suggested assay procedure is described.

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Black rot of crucifers continues to be a serious problem in spite of our knowledge of the epidemiology of the disease (2, 3, 6, 7, 13, 14). Perhaps the most important factor favoring epidemics of black rot is the ability of the pathogen to invade seed (5). Early workers realized the pathogen was seed-borne and investigated methods of eradication (3, 5). Hot-water soaks were shown to eliminate the pathogen (4) and were generally recommended. The need for treating each lot of seed or the effectiveness of each treatment has not, however, been determined. Because a single diseased plant may produce sufficient inoculum for an epidemic, control of black rot may depend upon detecting very low numbers of the pathogen in seed. To identify lots containing diseased seed, a simple, sensitive, and specific laboratory test is needed. Recently, several laboratory tests for detecting *X. campestris* in crucifer seed have been reported (1, 11, 12). In fact, one technique detected 0.3% diseased seed in several commercial seed lots which had been hot-water treated (12). Still, the sensitivities of these tests were not determined. In addition, the latter technique requires 2-3 weeks. The purpose of this study was to develop a laboratory test capable of detecting low numbers of *X. campestris* in crucifer seed and to compare the newly developed technique with other known techniques. A preliminary report of this investigation has been published (10).

MATERIALS AND METHODS.—*Bacterial isolate and seed inoculation.—Xanthomonas campestris* isolate B-24 was grown as previously described (8). Cabbage seeds were disinfested in a 20-ml disposable syringe with a wire screen placed in the bottom of the chamber to prevent seeds from escaping. After adding 4.5 g seed, a 1:75 dilution of Micro detergent (International Products Corp., Trenton, N. J.) was drawn into the syringe and the seeds agitated by shaking several times. After 20 minutes at room temperature, the detergent was removed and the seeds washed three times with sterile distilled water. The seeds were then soaked for 3 minutes in a 1% solution of sodium hypochlorite, washed three times with sterile distilled water, and placed in petri plates in a laminar-flow hood to dry. Seeds to be inoculated were placed on a double-stick tape in a petri plate. A small hole was made in the seed coat with a sterile needle and 1 μliter of a suspension of *X. campestris* was added. The inoculum was immediately infiltrated into the seed by vacuum. The number of viable cells of *X. campestris* in the inoculum was determined by assaying a 1-μliter sample on each of 10 plates of Difco nutrient agar (NA) as described (8). In no case did the inoculum contain more than 100 viable cells per μliter.

Media.—The nutrient-starch-cycloheximide agar (NSCA) which was used contained 10 g Difco nutrient agar, 10 g soluble starch (J. T. Baker Co.), and 250 mg cycloheximide (UpJohn Co.) per liter of distilled water. All ingredients were added prior to autoclaving. A solid medium selective for *X. campestris* (SX agar) was prepared as previously described (8).

RESULTS.—Following preliminary tests of several techniques, our efforts were directed toward improving agar plating techniques. Two plating techniques which

TABLE 1. Comparison of isolation frequency of *Xanthomonas campestris* from cabbage seed lots assayed by water enrichment only and direct plating after enrichment

Experiment no.	Inoculum[a]			Seed lots yielding *X. campestris* by[b] (no.)		
	Samples (no.)	Cells (mean no.)	Samples with *X. campestris* (no.)	Enrichment only on:		Direct plating after enrichment on:
				NSCA	SX	SX
1	10	1.4	1	0	1	1
2	10	1.6	1	0	0	1
3	10	6.3	4	1	2	4
4	10	76.4	9	8	8	9

[a]Ten, 1-μliter samples assayed on separate nutrient agar plates for each experiment.

[b]One seed inoculated with 1 μliter of inoculum was added to each of 10 lots of 100 seed. Each lot of seed was enriched in 1.0 ml water for 24 hours at 30 C before assaying two drops of liquid on nutrient-starch-cycloheximide agar (NSCA) and SX agar (9). All seeds were dried then placed on SX agar using a vacuum seed spotter. No diseased seedlings were observed from 1,000 noninoculated seeds sown in steam-sterilized soil.

appeared promising were: a modification of Andersen's enrichment assay (1), using NSCA or SX agar in place of NA, and the direct plating of enriched seeds on SX agar. To compare the two techniques, a single inoculated seed was added to each of 10 lots of 100 disinfested seeds in a petri plate. The seeds were enriched in 1.0 ml water for 24 hours at 30 C and two drops of liquid assayed on NSCA and SX agar, as described (8). After the remaining liquid was discarded, each lot of seed was air dried in a laminar flow hood before being placed on SX agar using a 100-hole vacuum seed spotter. The experiment was repeated four times using different sources of seed and inoculum. The mean number of viable cells of *X. campestris* per 1 μliter and the number of inoculum samples which contained the bacterium were determined for each experiment. Results (Table 1) showed that 15 of the 40 samples of inoculum (10 samples for each experiment) contained *X. campestris*. All 15 infested lots were detected by plating enriched seeds on SX agar. Using the enrichment only technique, nine infested lots were detected on NSCA and 11 were detected on SX agar. No diseased seedlings resulted from 1,000 noninoculated seeds from each source sown in steam-sterilized soil.

Similar results were obtained when a single inoculated seed (same batch of inoculated seed used for experiment 4, Table 1) was added to each of five lots of 1,000 seed. The pathogen was detected in all five lots when enriched seeds were placed on SX agar whereas no lots were detected when the enriched liquid was assayed.

That naturally diseased seed could be detected by plating enriched seeds on SX agar was established by finding *X. campestris* in a commercial cauliflower seed lot which originated from Japan. To determine the percent of black rot infestation, 4.5 g of the seeds were surface disinfested and assayed by plating 100 seeds directly on each of 10 SX agar plates. *X. campestris* was detected in 8 seeds. The identity of the eight isolates of *X. campestris* was confirmed by pathogenicity tests (9).

DISCUSSION.—Few laboratory tests have been devised to determine the presence of *Xanthomonas campestris* in crucifer seeds. Shackleton (11) and Srinivasan et al. (12) outlined sowing tests for detecting *X. campestris* in cabbage seed. After untreated seeds were placed on wet blotters (11), or aureofungin-treated seeds on water agar (12), seedlings were examined for symptoms of black rot. However, no tests were conducted to determine the minimum number of *X. campestris* cells per seed required for detection. In addition, as pointed out by Andersen (1), sowing tests are a disadvantage because of their failure to detect the pathogen in nongerminating seeds, and by requiring 2-3 weeks' time. To overcome these disadvantages, he (1) developed an enrichment technique that was successful in detecting naturally diseased seed of *Brassica oleraceae* var. *botrytis*. However, he also did not determine the sensitivity of the assay.

The success of our plating technique in detecting small numbers of *X. campestris* was due primarily to the use of cycloheximide and the assay of surface-disinfested seed. The addition of cycloheximide to our plating media not only inhibited fungi, but also seed germination. Thus, cycloheximide made it possible to exploit the ability of *X. campestris* to cause a soft rot.

A major disadvantage of the enrichment plating technique was that with some seed lots, surface disinfestion failed to sufficiently eliminate contaminating bacteria. Thus, when plated on NSCA or SX agar the enriched seeds were overgrown with other bacteria, thereby preventing any possible detection of *X. campestris*. Although we have experienced such conditions in only five of nearly 20 seed lots, the problem does exist. If such seed lots are found the seeds should not be enriched, but should be disinfested and placed directly onto NSCA or SX agar.

The following procedure for detecting black rot-diseased seed is recommended. To assay 1,000 seed, disinfest one sample [4-5 g (depending upon the size of seed)] of seed. After enriching in 2.0 ml water for 24 hours at 30 C, remove excess liquid with a needle and syringe and dry in a laminar-flow hood. Place 100 seeds on each of 10 plates of SX agar and incubate in the dark at 30 C. Record results after 5 and 10 days. Seeds surrounded by gray to purple, mucoid starch-hydrolyzing colonies should be recorded as positive. Representative colonies should be screened by streaking onto yeast extract–$CaCO_3$ agar (8) and observing the characteristic yellow, mucoid colonies. Finally, identity of *X. campestris* should be confirmed by pathogenicity tests, as previously described (9). If enriched seeds become overgrown by other bacteria, then a second 1,000-seed sample should be assayed by plating surface-disinfested seeds directly on SX agar.

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