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COMPLEMENTARY BACTERIAL ENRICHMENT TECHNIQUES FOR THE DETECTION OF *PSEUDOMONAS SYRINGAE* PV. *TOMATO* AND *XANTHOMONAS CAMPESTRIS* PV. *VESICATORIA* IN INFESTED TOMATO AND PEPPER SEEDS

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A scheme for routine seed testing for *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* in pepper and tomato seeds was developed. The scheme is based on different bacterial enrichment techniques. As few as 1000 and 10-100 colony forming units per gram of seeds were detected using a liquid enrichment technique or leaf enrichment technique, respectively. Relatively large amounts of saprophytes on the seed surfaces did not interfere with the detection of the pathogens.
KEY WORDS: Bacterial speck of tomato; bacterial scab of pepper; seedborne pathogens; phytopathogenic bacteria.

INTRODUCTION

The production of tomato and pepper seeds free of the pathogenic *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria* is of great economic importance for both the seed industry and the growers. It is commonly agreed that detecting small numbers of phytopathogenic bacteria in seeds is difficult, due to the relatively large numbers of saprophytic bacteria which accompany the pathogens on the seeds and interfere with the detection, due to their fast growth on selective media (12, 15). The methods and procedures developed to overcome this problem were reviewed recently (9, 10).

Tomato and pepper plants which germinate from seeds infested with *P. syringae* pv. *tomato* and *X. campestris* pv. *vesicatoria* may develop disease symptoms and initiate an epidemic in the field, if the environmental conditions are suitable (1, 3, 5, 8, 20).

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Heat treatment and fermentation for tomato seeds (2, 5) and chlorine treatment for pepper seeds (4) are commonly practised in the elimination of these pathogens. However, efficient quality control of the disinfestation process depends on the methods used to identify the pathogens within the host seeds and on their surfaces. Recently, a sensitive method was developed to detect very small numbers of *P. syringae* pv. *tomato* and *X. campestris* pv. *vesicatoria* in tomato and pepper seeds (15). The method is based on the selective enrichment of the pathogens inside their living host leaves. The sensitivity of the method is within the range of 10-100 bacteria per gram of seeds. The main drawback to this method is that only leaves of plants absolutely pathogen-free can be used. An additional difficulty, although a minor one, is that the tomato or pepper plants must be grown at least 6 weeks before the test. Therefore, a conventional laboratory technique for routine work is still needed. In this study, we describe combined bacterial enrichment techniques (leaves or liquid media) for routine seed testing for *X. campestris* pv. *vesicatoria* and *P. syringae* pv. *tomato* in pepper and tomato seeds.

MATERIALS AND METHODS

Organisms, Growth Conditions and Inoculum Preparation

Pseudomonas syringae pv. *tomato* (PST) (WT-1, WT-2, WT-3, Bet Dagan 134.1 and ATCC 10852), *Xanthomonas campestris* pv. *vesicatoria* (XCV) (R-2, R-3, Bet Dagan and ATCC 11633), tomato plants *(Lycopersicon esculentum)* cv. 'VF-198' (susceptible to bacterial speck, ref. 19) and pepper plants *(Capsicum annuum)* cv. 'Ma'or' were grown as described previously (1).

Cultures used as inoculum were grown on a yeast peptone liquid medium, containing 0.06 M potassium phosphate buffer, pH 6.8, in a shaking bath (100 strokes/min) at 30°C for 24 h. The cultures were centrifuged at 12,000 *g* for 10 min, washed three times in the same buffer, and finally adjusted to 0.3 absorbance units at 540 nm (for PST) and to 0.1 absorbance units at 420 nm (for XCV) in a Junior II Coleman spectrophotometer, corresponding to 10^9 colony-forming units (CFU) per ml.

Media

The following media were used:

Nutrient broth (Difco), 8 g per liter distilled water, for inoculum preparation and total bacterial count.

Nutrient-sodium deoxycholate (ND), containing 23 g nutrient agar (Difco) and 200 mg sodium deoxycholate per liter (applied after autoclaving), to count XCV from infested pepper seeds and leaves.

King-B-Fuchsin-TTC (KFT), containing King-B medium (6), 9 mg of basic fuchsin and 1.4 mg triphenyltetrazolium chloride (Sigma), to count PST from tomato seeds and leaves.

Liquid King-B medium (K), for enrichment of PST.
Phosphate buffer – sodium deoxycholate (PBD), containing phosphate buffer, pH 6.8, with 200 mg sodium deoxycholate, was used for enrichment of XCV in pepper seeds.

Leaf and Seed Disinfestation

Prior to inoculation, leaves were washed under a stream of tap water for 10 min, soaked for 3 min in a 0.5% solution of sodium hypochlorite (Frutarom, Israel), and washed again for 10 min under a stream of tap water. Seeds were soaked for 5 min in 2% of the same solution and washed three times with sterile distilled water. Seeds were placed in sterile petri dishes and allowed to dry for 3 h in a laminar flow hood.

Seed Inoculation

Surface-disinfested seeds or commercial seeds were infested by placing 10 g of seeds in 200 ml bacterial suspensions (10^1 to 10^9 cells/ml) under vacuum. The vacuum was broken abruptly to favor penetration of the pathogens into the seed cavities. PST was used for tomato seeds and XCV for pepper seeds. Seeds were dried as described above.

Leaf Inoculation

After surface disinfestation the leaves were placed aseptically on 0.5% water agar plates with their bottom side up. The leaves were inoculated with a 2-ml suspension of previously infested dried seeds in sterile distilled water as described below. The petri dishes were sealed with parafilm to prevent drying and cross contamination, and incubated under continuous light (100 W/m^2) at 28°C.

Liquid Enrichment

Seed samples (4000 seeds each) were soaked in the liquid King-B medium at 28°C in Erlenmeyer flasks and shaken vigorously for 4 h in a shaking bath (250 strokes/min).

Determination of Bacterial Population from Leaves and Seeds

Viable bacterial counts from soaked seed suspensions were estimated by spreading 0.2 ml of a ten-fold dilution (17) with a glass rod on agar plates with ND or KFT media. Total bacterial counts were performed on nutrient agar plates. Colonies were counted after 48 h of incubation at 28°C.

Colony Identification

Colonies suspected of being XCV were typically yellow and mucoid on yeast extract-dextrose-$CaCO_3$ agar (18). Colonies suspected of being PST had wrinkled margins and produced a typical fluorescent pigment. All PST colonies were oxidase-negative (&) Saprophytic pseudomonads were oxidase-positive and grew faster than

the parasitic species. After 24 h the saprophytes formed strongly fluorescent colonies with smooth edges (15). Suspected colonies were tested for pathogenicity as follows:

Two plants with 4-6 true leaves for each isolate were sprayed with a washed bacterial suspension (10^9 CFU/ml, supplemented with 0.1 g carborundum, 300 grid) and incubated separately in partial mist chambers (5 sec mist every 15 min, at 22±2°C for PST or 30±2°C for XCV for 6 or 10 days, respectively (1, 5).

All experiments were repeated two or three times in four replicates each.

RESULTS AND DISCUSSION

A scheme developed to detect XCV and PST in pepper and tomato seeds should fulfill the following requirements: *(a)* It should be sensitive enough to detect as small a number as possible of the phytopathogenic bacteria; *(b)* it should require little technical expertise or equipment and be relatively inexpensive; and *(c)* results should be obtained within a few days (9, 11, 13, 14, 16).

Figures 1 and 2 show that by using the leaf and the liquid enrichment techniques as few as 10-100 and 1000 CFU/g seeds, respectively, were detectable. (Compare the left and the right columns in each pathogen infestation level.) No decrease occurred in pathogen detection from infested seeds which had not been previously disinfested. PST colonies were detectable on the KFT diagnostic plates in spite of the fact that large numbers of saprophytic bacteria (up to 10^8 CFU/g seeds) developed at the same time. The interference of saprophytic bacteria in detection of XCV was minimal due to the supply of antibiotics to either the enrichment or growth medium. The typical yellow pigment of XCV also helped to distinguish it from other saprophytes. Similar trends in the results were obtained from all isolates tested.

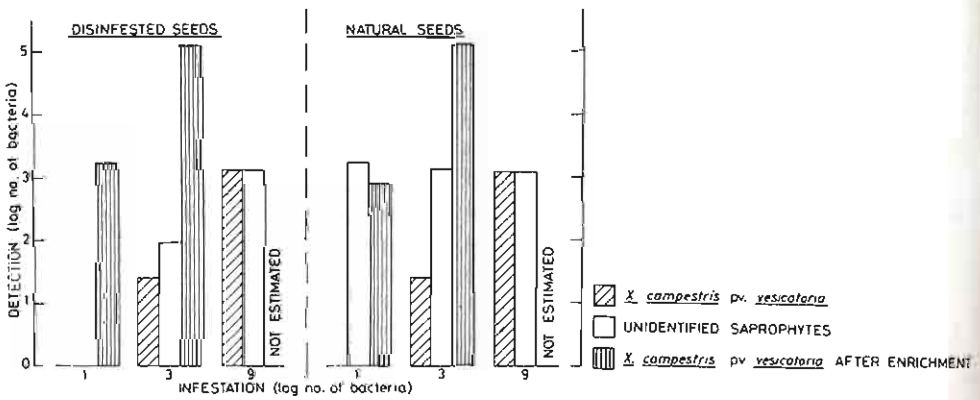

Fig. 1. Detection of *Xanthomonas campestris* pv. *vesicatoria* in pepper seeds. PBD, incubation medium; ND, count method.

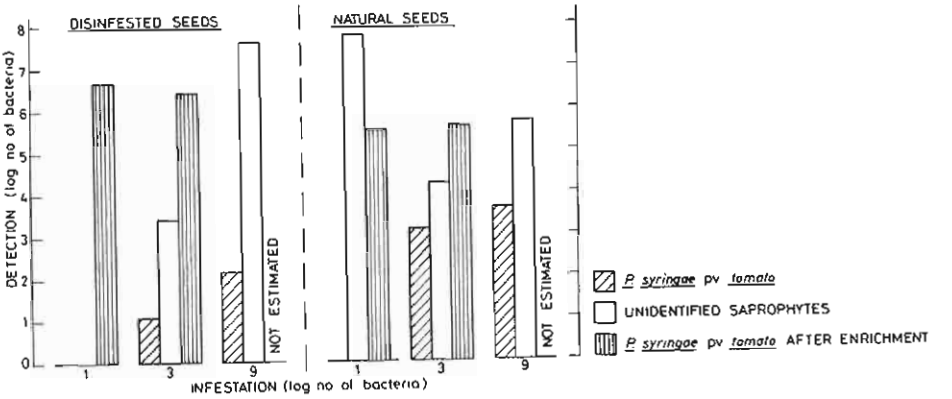

Fig. 2. Detection of *Pseudomonas syringae* pv. *tomato* in tomato seeds. K, incubation medium; KFT, count method.

In spite of the heavy saprophytic interference, the ten-fold dilutions of the enriched suspensions used for the detection of both bacteria should be only 10^{-1} or 10^{-2}. In higher dilutions, the pathogens were not detectable. Naturally, the numbers of saprophytes given in Figs. 1 and 2 were counted by the conventional dilution technique.

The sensitivity of the medium enrichment techniques was 10^3 CFU/g seeds and it was found suitable for routine inspection of highly contaminated seed lots. However, in the few cases where only small numbers of pathogenic bacteria existed in the seeds, a leaf enrichment method which is more precise but more complicated for routine work was used. It was found that when seeds were inoculated with suspensions containing 10 CFU/ml, the medium enrichment technique did not detect the few bacterial cells. However, after enrichment of the same suspensions in the compatible host leaves, both pathogens could be detected easily. The recommended assay for detection of PST and XCV is given in Fig. 3.

Thirty-eight commercial pepper lots and 53 tomato lots (200 g each) were tested by the liquid enrichment method for the presence of the compatible pathogens. All samples were contaminated with saprophytic bacteria at levels higher than 10^8 CFU/g seeds. Seven pepper lots and 28 tomato lots were contaminated with the compatible pathogen at levels higher than 10^3 CFU/g seeds. The remaining lots were tested by the leaf enrichment method and it was found that eight pepper lots and five tomato lots were contaminated with the compatible pathogen at low concentration.

These enrichment techniques are inexpensive, simple and reliable and have

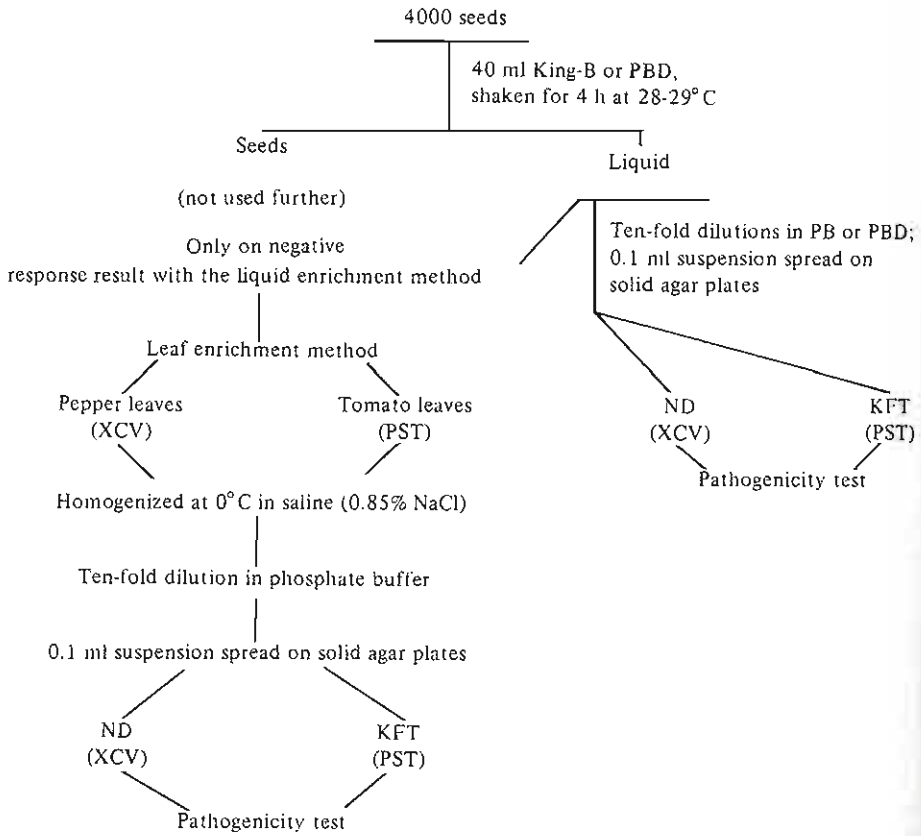

Fig. 3. Flow diagram for the detection of *Pseudomonas syringae* pv. *tomato* (PST) and *Xanthomonas campestris* pv. *vesicatoria* (XCV) in tomato and pepper seeds by bacterial enrichment techniques.

been run successfully in our laboratories by non-expert technicians during the last 2 years, since they were developed. They are therefore recommended for routine seed inspection.

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