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Detached leaf enrichment: a method for detecting small numbers of *Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria* in seed and symptomless leaves of tomato and pepper

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A method for detecting 10^1–10^2 cells of phytopathogenic bacteria (*Pseudomonas syringae* pv. *tomato* and *Xanthomonas campestris* pv. *vesicatoria*) in either tomato or pepper seed was developed. The method is based on the enrichment of the compatible pathogen inside a detached leaf of its host when placed on a water agar medium. It was found to be superior to the diagnostic growth media method commonly used and to permit the detection of the pathogens in symptomless plants.

The production of vegetable seed free of pathogenic bacteria is of great economic importance for both the seed industry and the growers. Bacterial diseases of many plants are seed borne.

Plants which germinated from seed externally infested with *Pseudomonas syringae* pv. *tomato* or *Xanthomonas campestris* pv. *vesicatoria*, the causal agents of bacterial speck and scab of tomato and pepper, respectively (Crossan & Morehart 1964; Devash *et al.* 1980), may develop disease symptoms and initiate an epidemic in the field under favourable environmental conditions (Bashan *et al.* 1978; Crossan & Morehart 1964; Devash *et al.* 1980; Lewis & Brown 1961; Okon *et al.* 1979; Yunis *et al.* 1980b, 1981). To date, the detection of small numbers of phytopathogenic bacteria in and on seed has been difficult because of the relatively large numbers of saprophytic bacteria, taxonomically related to the pathogens, which accompany the pathogens and interfere with their growth on selective media (Schaad & White 1974).

The methods and procedures developed to overcome this problem have recently been reviewed by Neergaard (1977) who noted that most procedures do not detect small numbers of pathogenic bacteria. In addition, as pointed out by Weller & Saettler (1980), detection of infection with *Xanthomonas phaseoli* in symptomless seed would be almost impossible by current test methods because of the low frequency of the organisms in seed lots and their low population level within an individual seed (less than 10^5 cells/bean seed).

Immersion of tomato seed in hot water (52°C for 1 h) eliminated *Ps. syringae* pv. *tomato* without damaging the seed (Devash *et al.* 1980). This treatment, however, could not be used with pepper seeds because of their susceptibility to high temperatures. In Israel at present, no treatment is used against pathogenic bacteria in

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pepper seed. Development of seed disinfestation methods depends upon sensitive bacterial testing methods.

Populations of pathogenic and non-pathogenic bacteria develop in typically different patterns in plant tissue. Compatible pathogens multiply rapidly but although incompatible pathogens also multiply their final numbers are lower (Allington & Chamberlain 1949; Diachun & Troutman 1954; Ercolani & Crosse 1966; Stall & Cook 1966; Omer & Wood 1969; Young & Paton 1972; Young 1974; Brown 1980; Daub & Hagedorn 1980). Klement & Lovrekovich (1961) and Klement *et al.* (1964) demonstrated and suggested the concept that saprophytic bacteria did not multiply in leaf tissue under normal plant growth conditions.

Based on the above observations Kennedy (1969) and Goto (1972) were able to detect small numbers of *X. citri* and *Ps. glycinea* respectively from soil, by injecting a mixture of pathogens and saprophytes into leaves of healthy plants. It is also noteworthy that fluorescent antibody methods can be used for the same purpose, but results can be difficult to interpret (Neergaard 1977).

The purpose of this study was to develop a simple, reliable method for detecting low levels of *Ps. syringae* pv. *tomato* and *X. campestris* pv. *vesicatoria* in tomato and pepper seed, by using the host leaves as a pathogen enrichment medium. Preliminary reports of this method have been presented elsewhere (Henis *et al.* 1980; Sharon *et al.* 1981).

Materials and Methods

ORGANISMS, GROWTH CONDITIONS AND INOCULUM PREPARATION

Pseudomonas syringae pv. *tomato* (W T-1) (specific to tomato plants), *X. campestris* pv. *vesicatoria* (R-3) (specific to pepper plants), tomato plants (*Lycopersicon esculentum*) cv. 'VF-198' (susceptible to bacterial speck; (Yunis *et al.* 1980a) and pepper plants (*Capsicum annuum*) cv. 'Ma'or' were grown as previously described (Bashan *et al.* 1978). Inoculum was prepared from 24 h liquid cultures according to Okon *et al.* (1978). All experiments described in this study were carried out with either *Ps. syringae* pv. *tomato* on tomato seed and leaves or *X. campestris* pv. *vesicatoria* on pepper seed and leaves.

MEDIA

Nutrient broth (Difco), 8 g/l was used for inoculum preparation. Nutrient sodium deoxycholate (ND) containing 23 g/l nutrient agar (Difco) and 0·15 g/l sodium deoxycholate was used to count *X. campestris* pv. *vesicatoria* from infested pepper seed and leaves. King-B-fuchsin-TTC (KFT) containing King-B-medium (King *et al.* 1954), 9 μg/ml fuchsin and 14 μg/ml triphenyltetrazolium chloride (Sigma) was used to count *Ps. syringae* pv. *tomato* from tomato seed and leaves. Medium D5 (Kado & Heskett 1970) containing (g/l): cellobiose 10; KH_2PO_4 3; NaH_2PO_4 1; NH_4Cl 1; and $MgSO_4 \cdot 7H_2O$ 0·3; or the medium of Schaad & White (1974) containing (g/l): starch 10; beef extract 1; NH_4Cl 5; KH_2PO_4 2; and 1 ml of a 1% w/v solution in 20% (v/v) ethanol of methyl violet B, 2 ml of a 1% (w/v) solution methyl green, and 250 mg of cycloheximide (SX) were also used to count *X. campestris* pv. *vesicatoria* from infested pepper leaves.

LEAF AND SEED DISINFESTATION

Before inoculation the leaves were washed under a stream of tap water for 10 min to remove dust and dirt, soaked for 3 min in a 0·5% (w/v) solution of sodium hypochlorite (Frutarom, Israel) and washed again for 10 min under a stream of tap water. The natural phyllosphere microflora of leaves was reduced 10-fold and no toxic effect of the NaOCl was observed on leaves after 8 days. Seed were soaked for 5 min in 1% of the same solution and washed three times with sterile distilled water. Seed were placed to dry in sterile Petri dishes in a laminar flow hood for 3 h.

SEED INOCULATION

Surface sterilized or untreated commercial seeds were infested by shaking 1 g of seed for 10 min in 10 ml of bacterial suspension (1×10^1–1×10^7 cfu/ml). Seed were dried as described above.

LEAF INOCULATION

After surface disinfestation the leaves were placed aseptically on 0·5% (w/w) water agar plates with their adaxial side up. The leaves

were inoculated by spreading 2 ml amounts of a bacterial suspension of either pure cultures of each bacterium or suspensions made from previously infested dried seed maintained in phosphate buffer 0·06 mol/l, pH 7·0, for 2 h. Numbers of bacteria on seed were 10^1–10^2 cfu/g seed as calculated by extrapolation from Fig. 1.

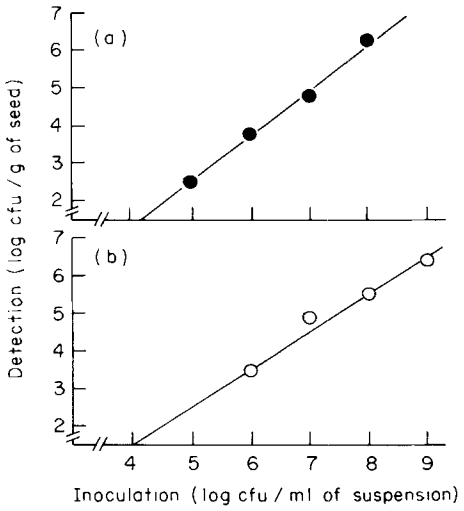

Fig. 1. Survival of (a) *Xanthomonas campestris* pv. *vesicatoria* and (b) *Pseudomonas syringae* pv. *tomato* on dried tomato and pepper seeds, respectively.

Disinfested control leaves were treated with phosphate-buffer, incubated under the same conditions and tested for leaf surface and endogenous bacteria. The Petri dishes were sealed with Parafilm to prevent drying and cross contamination. They were incubated under continuous fluorescent light (10 000 lux) at 25 ± 2°C.

DETERMINATION OF BACTERIAL POPULATION FROM LEAVES AND SEED

Incubated leaves, about 1 g fresh weight, (24–96 h) were placed in 3·0% (w/w) sodium hypochlorite for 5 min, washed 3 times with sterile distilled water and homogenized in 20 ml of saline solution (8·5 g NaCl/l) in a sterile Omnimixer (Sorvall). Serially diluted suspensions (0·1 ml) were spread on solid agar plates (ND for *X. campestris* pv. *vesicatoria* and KFT for *Ps. syringae* pv. *tomato*). Colonies were counted after 48 h incubation at 30°C. For bacterial counts of the surface populations, leaves were shaken vigorously for 30 min in saline before disinfestation. Infested seed were soaked in sterile saline, shaken vigorously for 30 min, diluted and plated as described above. Estimation of numbers of bacteria were done by ten-fold dilution series (Taylor 1962).

Pathogenicity tests

These were done according to the method of Devash *et al.* (1980).

Results

SURVIVAL OF *Ps. syringae* PV. *tomato* AND *X. campestris* PV. *vesicatoria* IN DRY SEED

Surface disinfested dry tomato and pepper seeds were inoculated with different bacterial suspensions and dried. It was observed (Fig. 1) that after short periods, i.e. 5 d, the two pathogens survived well on the dry seed stored in Parafilm sealed Petri dishes. By using this procedure the number of bacteria recovered from seed surface was about 10-fold lower.

EVALUATION OF DIAGNOSTIC MEDIA

It was observed in preliminary tests that the surface of untreated pepper and tomato seed contained a large population of saprophytes. Therefore, the use of diagnostic media was essential. In ND medium, *X. campestris* pv. *vesicatoria* formed typical yellow, mucoid colonies and Gram positive bacteria were inhibited. On the other selective media based on cellobiose or starch, colonies of the pathogen did not develop their usual yellow pigmentation, making them indistinguishable from other Gram negative bacteria. After incubation in KFT medium for 72 h, *Ps. syringae* pv. *tomato* colonies developed as typical small colonies with wrinkled edges (Fig. 2a). On the other hand, saprophytic *Pseudomonas* colonies developed must faster, forming strongly fluorescent large colonies after 24 h (Fig. 2b). Other Gram-negative saprophytes formed red, non-fluorescent colonies (Fig. 2c).

The efficiency of the diagnostic media in the detection of *Ps. syringae* pv. *tomato* on tomato seed was tested using seed inoculated with suspensions containing 10^1–10^6 cfu/ml. It was

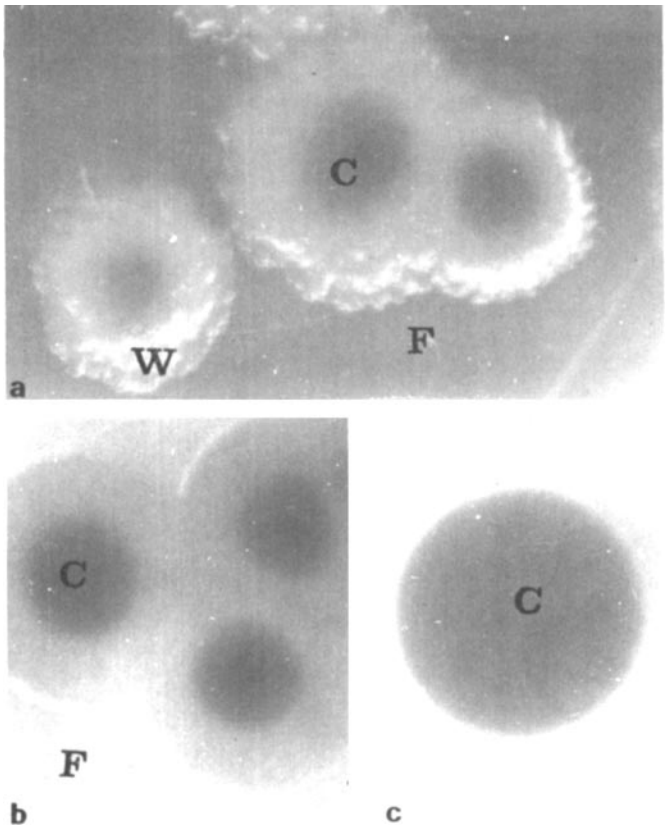

Fig. 2. Typical colonies that contaminated tomato seeds. (a) *Pseudomonas syringae* pv. *tomato*; (b) *Pseudomonas fluorescens*; (c) non-fluorescent Gram negative bacteria (× 40). C, colony; F, fluorescence; W, wrinkled.

clearly observed (Table 1) that the diagnostic media could detect the pathogens only in the tomato seed previously infested with more than 10^4 cfu/ml. Results were similar in pepper seed infested with *X. campestris* pv. *vesicatoria*. Therefore this method was deemed unsuitable to detect small numbers of these phytopathogens in their respective host's seed.

DEVELOPMENT OF PHYTOPATHOGENIC AND SAPROPHYTIC BACTERIA WITHIN AND ON THE SURFACE OF PEPPER LEAVES

Bacterial counts on the leaf surfaces and inside their tissues were followed daily. Both *X. campestris* pv. *vesicatoria* and *Ps. fluorescens* multiplied on the surface of pepper leaves, whereas

Table 1. Detection of *Pseudomonas syringae* pv. *tomato* in artificially infested tomato seeds using KFT medium

Seed treatment	Inoculum concentration (cfu/ml)	Detection	Minimal detection level (cfu/g of seeds)
Pre-disinfestation	8×10^6	+	$2{\cdot}6 \times 10^5$
	2×10^4	+	$2{\cdot}6 \times 10^3$
	$10^1 - 10^3$	–	—

Experiments were carried out three times. Values are means of one experiment. Each inoculum level had four replicates and each replicate was spread on five KFT agar plates. Seeds were disinfested by soaking them for 1 h in a water bath at 52°C (Devash *et al.* 1980).

only the plant pathogenic *X. campestris* pv. *vesicatoria* multiplied inside the pepper leaves (Fig. 3). Similar results were obtained with *Ps. syringae* pv. *tomato* and *Ps. fluorescens* on tomato leaves.

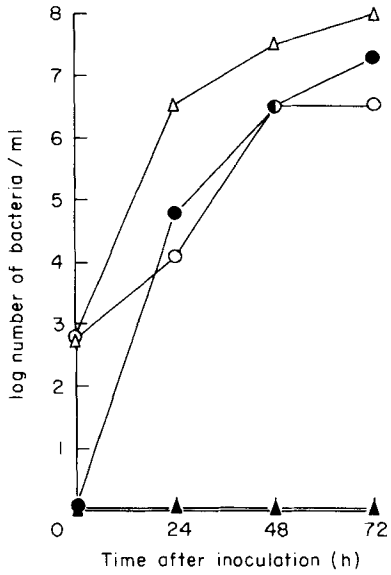

Fig. 3. Multiplication of *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas fluorescens* within pepper leaves and on their surface. ○, *X. campestris* pv. *vesicatoria* on the leaf surface; ●, *X. campestris* pv. *vesicatoria* within the leaf; △, *Ps. fluorescens* on the leaf surface; ▲, *Ps. fluorescens* within the leaf.

ENRICHMENT OF BACTERIA USING HOST LEAVES

Preliminary observations showed that placing artificially infested undetectable 'diseased' seed (as detected by diagnostic medium) in a drop of water on the host leaf for 2 days was followed by a selective multiplication of the pathogen in the leaf after the seeds were removed. Also, typical symptoms of the two diseases developed within 6–8 days in the leaves inoculated with suspensions obtained from the seed, indicating that the seeds were contaminated with pathogens. Drops of either healthy seed homogenate or leaf extract (2 g of seeds or leaves homogenized in an Omni-mixer in 5 ml of phosphate buffer) placed on pepper leaves had no effect on the leaves after 8 incubation.

Within 4–5 days typical speck or scab symptoms could be observed. Furthermore, selective pathogen multiplication inside the host increased with time and could first be detected within 24 h. On diagnostic media bacterial counts were already possible with minimal disturbances of accompanying saprophytes (increasing from 0 before inoculation to 10^2 cfu/ml after 24 h). The growth of pathogens was unaffected by possible toxic compounds produced by the diseased leaves.

Using this method only leaves of pathogen-free greenhouse plants with no previous history of bacterial disease should be used for enrichment. Another positive feature of the system was that the leaves placed in such water-agar plates remained intact and were not contaminated with tissue-degrading micro-organisms. Preliminary identification of bacteria according to their morphological characteristics could be carried out on agar after as short a time as 24–48 h. To test the capacity of this method to detect low numbers of pathogenic bacteria in seed, leaves were inoculated with 2 ml amounts of the suspensions obtained from previously inoculated seed. The experiment was repeated 10 times with each pathogen. In all cases enrichment of the compatible pathogen developed from 0 (non-detectable) at time of inoculation to 10^7 cfu *Ps. syringae* pv. *tomato*/g leaf in tomato and 10^6 cfu *X. campestris* pv. *vesicatoria*/g leaf in pepper, after 96 h incubation. Saprophytic bacteria were not enriched inside the tissues. No pathogens could be detected in leaves inoculated with suspensions obtained from non-infested seed.

DETECTION OF SEED-BORNE PHYTOPATHOGENIC BACTERIA BY THE LEAF ENRICHMENT METHOD AS COMPARED WITH THE DIAGNOSTIC MEDIA METHOD

Eight 200 g commercial lots of tomato seed and eight of pepper seed, grown in different symptomless seed production fields in various parts of Israel were tested for the presence of *Ps. syringae* pv. *tomato* or *X. campestris* pv. *vesicatoria*, respectively, using either the leaf enrichment or the diagnostic media methods. Three replicate samples, each of 1000 seeds from every lot were tested. In all samples saprophytic bacteria reached levels higher than 10^8 cfu/g of seed. With diagnostic media it was not possible to

detect any pathogens in every sample. With the leaf enrichment method, however, 90% of pepper seed lots were found to be contaminated with *X. campestris* pv. *vesicatoria*, whereas 50% of tomato seed lots were contaminated with *Ps. syringae* pv. *tomato*. The pathogens isolated were tested for pathogenicity, and their identities confirmed bacteriologically.

DETECTION OF BACTERIAL SPECK OR SCAB PATHOGENS IN SYMPTOMLESS LEAVES

Surface disinfested leaves obtained from 3-month-old symptomless pepper and tomato plants, grown in a greenhouse with previous history of bacterial diseases, were incubated on water agar as described above. After 72 h incubation it was possible to detect pathogens inside the host leaves (10^6 cfu/g leaf). Pathogenicity of the organisms isolated from leaves was confirmed, and their identities verified.

After 4–8 days all leaves showed typical speck (tomato) or scab symptoms (pepper; Fig. 4a). In cases of doubtful disease symptoms leaves were surface disinfested and placed on nutrient agar or KFT plates for 5 h. The leaves were then removed and the plates incubated for 48 h at 30°C. On the leaf print obtained by the procedure it was possible to locate the few infected leaf sites (Fig. 4b), with minimal disturbances of other saprophytes naturally present on the leaf surface.

Discussion

The method described in this paper is based on the selective ability of the pathogens to multiply inside their living host plant as compared with the bacteria which develop only on the surface (Klement & Lovrekovich 1961; Klement *et al.* 1964). Its simplicity and its high sensitivity in detecting very low population levels of pathogens in the range of 10–100 cfu/g of seed makes it suitable for use by seed producers and vegetable growers for the following purposes:

(i) preliminary screening for the presence of the pathogen on leaves taken from plants growing in fields designed for seed production, ruling out positive fields unless appropriate preventive treatment is applied;

(ii) routine seed checks throughout seed production to determine whether or not the expensive disinfection process is needed;

(iii) quality control of seed disinfestation;

(iv) early detection of bacterial speck or scab in fields and greenhouses, made possible because the method detects disease in symptomless leaves. Chemical control can then be given only when necessary, saving costly bacteriocides and lessening the environmental pollution caused by frequent sprays. Finally, the method perhaps could be extended to detect bacterial pathogens other than *Ps. syringae* pv. *tomato* and *X. campestris* pv. *vesicatoria*.

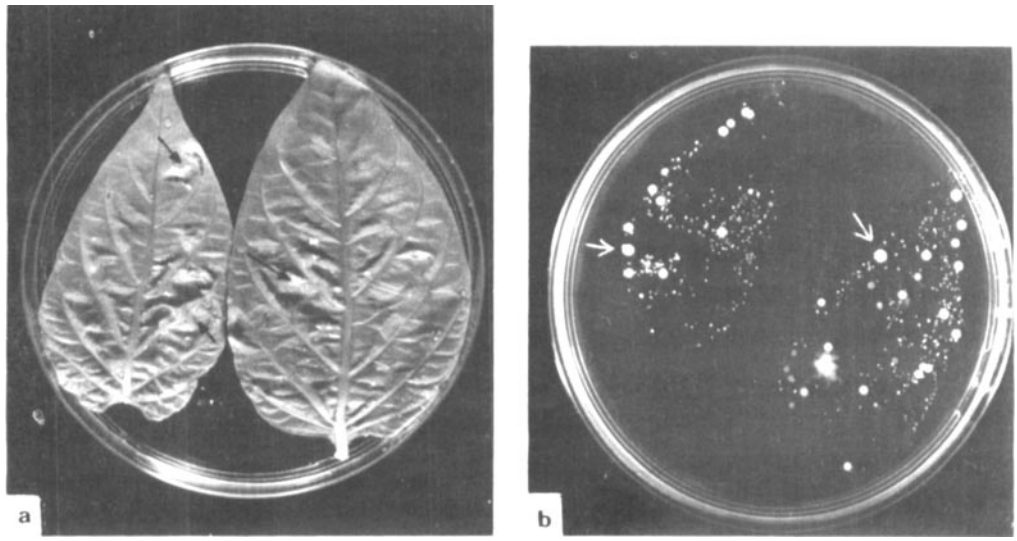

Fig. 4. (a) Enrichment system of *Xanthomonas campestris* pv. *vesicatoria* on pepper leaves incubated on water agar for 6 d at 25°C under continuous illumination. Arrows indicate visible symptoms. (b) Impression of pepper leaves on nutrient agar medium after 48 h incubation at 30°C. Arrows indicate colonies of *X. campestris* pv. *vesicatoria*.

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