

# Detection of *Pseudomonas syringae* in *Cucurbita pepo* (Squash) Seed

**MARCH 2022**

**Developed by ISHI-Veg**

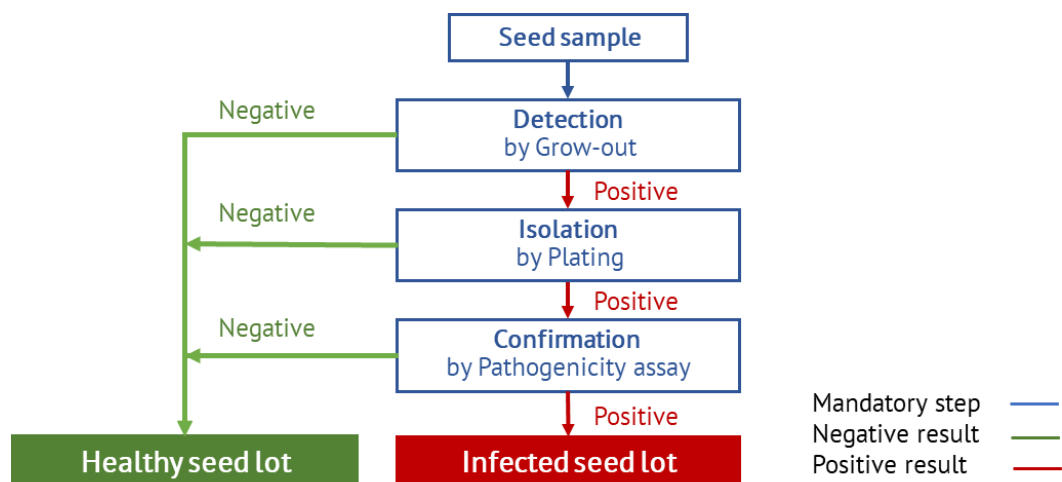
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## Detection of *Pseudomonas syringae* in *Cucurbita pepo* (Squash) Seed

**Crop:** Zucchini squash (*Cucurbita pepo*)  
**Pathogen(s):** *Pseudomonas syringae* causing ‘Zucchini vein clearing disease’.  
**Version:** 1 (March 2022)

### PRINCIPLE

Detection of infectious *Pseudomonas syringae* in seed of *Cucurbita pepo* (Zucchini squash) is done by growing out seeds under controlled environmental conditions followed by isolating the pathogen in pure culture from symptomatic seedlings and confirming its pathogenicity on plants by a pathogenicity assay. The method process workflow is presented in Figure 1.



**Figure 1.** Method process workflow.

### METHOD VALIDATION

This method has been validated according to the ISHI-Veg Guidelines for the Validation of Seed Health Test (Lybeert *et al.* 2022).

### RESTRICTIONS ON USE

Technical details on the reagents / material used in the validation study (e.g. supplier information) are provided in the protocol and the validation report. If material and consumables from different suppliers are used, it is necessary to verify their performance.

This test method is suitable for untreated seed.

This test method is suitable for seed that has been treated using physical (e.g. hot water) or chemical (acid extraction, calcium or sodium hypochlorite, tri-sodium phosphate, etc.) processes with the aim of disinfestation/disinfection, provided that any residue, if present, does not

influence the assay. It is the responsibility of the user to check for inhibition by experimental comparisons or other means.

This test method has not been validated for seed treated with protective chemicals or biological substances. If treated seed is tested using this method, it is the responsibility of the user to determine empirically (through analysis, sample spiking, or experimental comparisons) whether the protective chemicals or biological substances have an effect on method results.

## **METHOD EXECUTION**

To ensure process standardization and valid results, it is strongly recommended that the best practices developed by ISHI-Veg are followed. (see <https://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-method-development/>).

## **SAMPLE AND SUBSAMPLE SIZE**

The recommended minimum sample size is 1,000 seeds.

## **RATIONALE**

Grow-out-based testing is found appropriate to test squash seed lots for viable, infectious *P. syringae*, which are the causal agent of the “Zucchini vein clearing” disease.

## **REVISION HISTORY**

<b>Version</b>	<b>Date</b>	<b>Changes (minor editorial changes not indicated)</b>
1	March 2022	First version of the protocol

## Protocol for detection of *Pseudomonas syringae* in *Cucurbita pepo* (squash) seed

### I. DETECTION BY GROW-OUT ASSAY, ISOLATION BY PLATING AND CONFIRMATION BY PATHOGENICITY ASSAY

For grow-out and pathogenicity assays, in-house method optimization is often necessary by changing certain parameters as described in [Best Practices for Sweat Box and Grow-Out assays](#) and [Best Practices for Biological Assays in Seed Health Tests](#).

#### Materials

- NaCl solution (recipe see Table I.1)
- 70% (v/v) ethanol
- Controls (Table I.2)
- Growth chamber / Greenhouse compartment (25–30 °C with relative humidity  $\geq$  70 %)
- Laminar airflow cabinet
- Commercial sterilized or virgin potting mix
- Trays
- Healthy squash seeds
- Small grinding plastic bags and a press grinder (or equivalent)
- Forceps
- Scalpel
- Inoculation loop
- Cotton swab
- Petri dishes
- Microliter pipettes
- Plates of LBC + AL medium (recipe see Table I.3)
- Lab disposables

**Table I.1.** NaCl solution 0.85%.

Compound	Amount / L
Sodium chloride (NaCl)	8.5 g
De-ionized water up to a volume of	1 L

Note: Autoclave at 121 °C, 15 psi for 15 min.

**Table I.2.** Types of controls used.

Control type	Description	Assay
Positive Process Control (PPC)	A known <i>P. syringae</i> -positive seed sample	Grow-out
Negative Process Control (NPC)	A known <i>P. syringae</i> -negative seed sample	
Positive Control (PC)	A reference <i>P. syringae</i> strain	Pathogenicity assay
Negative Control (NC)	NaCl solution	

**Table I.3.** Composition of LBC+AL medium<sup>a</sup>.

Compound	Amount / L
Yeast extract	2 g
Bacto-peptone	5 g
Sucrose	50 g
Boric acid	1.5 g
Bacto agar	15 g
NaOH 1N	2 mL
Nystatin <sup>b</sup> (20 mg/mL in 50% DMSO/50% (v/v) ethanol)	20 mg (1.0 mL)
Cephalexin <sup>b</sup> (10 mg/mL in distilled or de-ionized water)	80 mg (8 mL)
Lincomycin <sup>b</sup> (50 mg/mL in distilled water)	50 mg (1 mL)
De-ionized water up to a volume of	1 L

<sup>a</sup> Setting of pH is not required

<sup>b</sup> Added after autoclaving (Temp < 50 °C)

Antibiotics stock solutions and other supplements prepared in distilled/de-ionized water must be sterilized using a 0.2 µm bacterial filter. Alternatively, add the antibiotic powder to the autoclaved distilled/de-ionized water. Solutions prepared in 70% (v/v) ethanol need no sterilization.

Storage conditions and duration may affect antibiotic activity, which can influence the test performance.

## 1. Grow-out

1.1. Prepare growth chamber or greenhouse compartment. Clean and disinfect all surfaces and equipment before starting the assay. Disinfect hands between seed samples sown. Separate trays from one sample to another to avoid cross-contamination.

1.2. Sow seeds, together with PPC and NPC (Table I.2), in a commercial sterilized or virgin potting mix and incubate for 48 hours at 25 °C for seedling emergence.

Seedling density should be such to allow unrestricted seedling development for a period of three weeks, corresponding to a maximum of 900 seeds per square meter

For a routine test, plants coming from a same seed lot can be close.

1.3. After seedling emergence:

Temperature and relative humidity should be uniform across the area where plants are raised. Maintain relative humidity at 70% or higher, from the time seedlings emerge to final reading. Relative humidity should not be lower than 50% for more than 12 hours.

Maintain temperatures at 25-30 °C during the day and 15-18 °C during the night until final inspection. Temperatures should not be out of this range for more than 12 hours. Record temperature and relative humidity, preferably above plant canopy for the duration of the test.

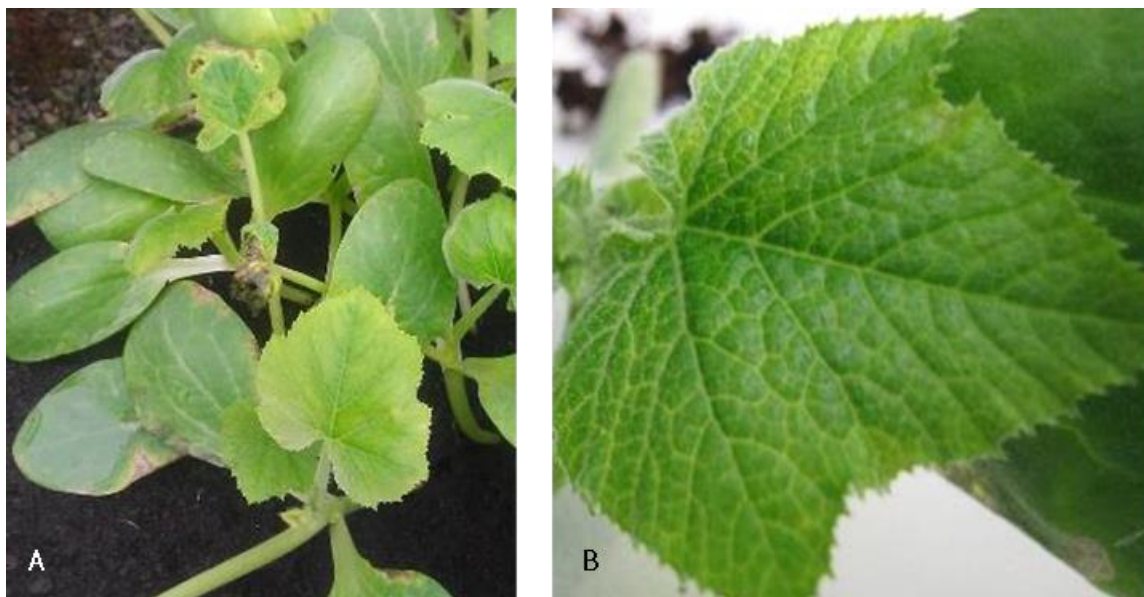
Supplement light for 12 hours per day, if necessary, in greenhouse. In a growth chamber, light should be moderate with respect to heat radiation.

Overhead watering, just sufficient to create uniform leaf wetness, shortly before onset of the daily cooler (night) period will speed up symptom development.

- 1.4. Inspect seedlings 11, 14 and 21 days after sowing. To avoid cross contamination, disinfect hands between seed samples during each observation. Final inspection is done when seedlings have two developed true leaves. In a growth chamber final inspection may be done earlier (14 days). Do not remove plants showing symptoms during the intermediate observation.

Symptoms typical of *Pseudomonas syringae* occur on cotyledons and true leaves: marginal necrosis and water-soaked to dark necrotic lesions (Figure I.1).

- 1.5. Record number of plants showing symptoms and number of plants observed per inspection days.



**Figure I.1.** Typical symptoms of *P. syringae* in a grow-out. In a greenhouse (A) and growth chamber (B).

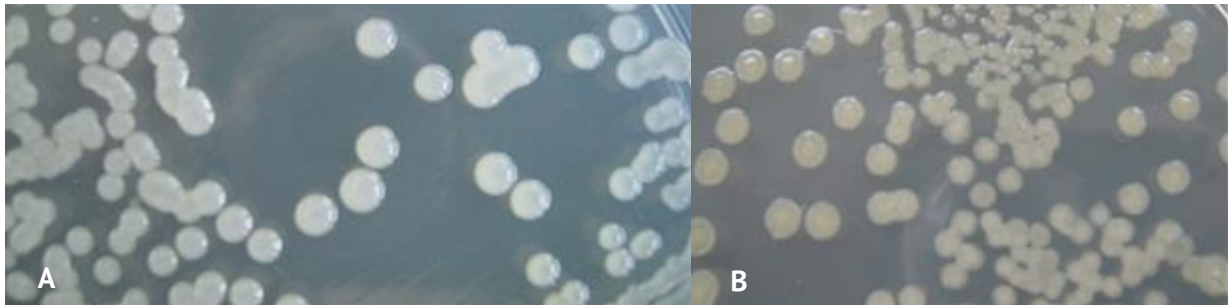
## 2. Isolation of the bacterium

- 2.1. Harvest plants showing symptoms individually in plastic bags: choose preferably plants showing typical symptoms. Harvest at least two suspected plant per subsample if over two plants show symptoms.

Note: When no plants with symptoms are found, the test result can be considered negative but only if the NPC and PPC give expected results. The PPC should give plants with symptoms, and the NPC should give no symptoms.

- 2.2. Put a droplet of sterile NaCl solution in an empty Petri dish.
- 2.3. Briefly disinfect the surface of the symptomatic leaf or cotyledon with 70 % (v/v) ethanol.
- 2.4. With forceps and a scalpel, excise a small piece of symptomatic tissue (marginal necrosis).

- 2.5. Mash the tissue in the droplet and wait for 15-30 min for bacteria to ooze from the pieces. Dip an inoculation loop in the droplet and streak on the surface of one LBC+AL plate. From the primary streak, make two more streaks on the same plate attempting to obtain individual colonies.
- 2.6. Incubate plates at 26-28 °C, read after 48 hours.
- 2.7. Two colony types can be expected for pathogenic strains: white translucent and mucous (Figure I.2A) and opaque and drier (Figure I.2B).



**Figure I.2.** *Pseudomonas syringae* colonies on LBC+AL: white, translucent and mucous (A) and opaque and drier (B).

- 2.8. Select the most dominant type of colony for pathogenicity testing (select one colony per harvested plant).
- 2.9. Transfer the colony on a plate of LBC+AL.
- 2.10. Proceed with the pathogenicity assay for confirmation.

### 3. Pathogenicity assay

- 3.1. For each suspect colony to be tested and for the PC and NC, grow four squash seedlings until the first true leaf just starts to develop (approximately one week after sowing).
- 3.2. Transfer suspect colonies by streaking to a fresh plate of LBC+AL and incubate for 48 hours at 26-28 °C. Include a known pathogenic strain of *P. syringae* as a positive control.
- 3.3. Harvest cells with an inoculation loop and suspend in NaCl solution to a concentration of  $10^6$ - $10^8$  cells/mL.
- 3.4. Inoculate the 4 assay plants by wetting the adaxial (upper) side of both cotyledons with a cotton swab soaked in the bacterial suspension.  
Inoculate the NC assay plant with NaCl solution.
- 3.5. Incubate inoculated seedlings for 7 days at 18-24 °C and 50-85% relative humidity.
- 3.6. Score for pathogenic reactions (+/-), which are characterized by necrotic spots on the cotyledons (Figure I.3).

A colony is considered as positive if at least one inoculated plant (out of the 4) shows symptoms.



A seed lot is considered as infected if at least one colony obtained from the suspected plants gives a positive result in the pathogenicity assay.

Note: Test results are only valid when all included controls presented in Table I.2 obtained expected result. At least one of the four test plants inoculated with the PC and PPC should develop clear symptoms. Test plants inoculated with the NC should develop no symptoms.



**Figure I.3.** Results of pathogenicity assay. Symptoms on inoculated cotyledons 7 days post-inoculation of pathogenic *P. syringae* grown in greenhouse at 45-60% relative humidity (A) and climate chamber at 85-95% relative humidity (B). Note the sunken pinpoint lesions and surrounding halos in A.

## REFERENCES

Lybeert, H., Delisle, J., Willmann, R.W., Eskes, C. & Woudenberg, J.H.C. (2022). Detection of *Pseudomonas syringae* in *Cucurbita pepo* (squash) seed by grow-out. Validation report, International Seed Federation (ISF), Nyon, Switzerland. <https://worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-validation-reports/>