International Rules for Seed Testing 2025

Validated Seed Health Testing Methods

7-023: Detection of *Pseudomonas savastanoi* pv. *phaseolicola* in *Phaseolus vulgaris* (bean) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2024 in Cambridge, United Kingdom

Effective from 1 January 2025

Validation reports

See References. Copies are available by e-mail from the ISTA Secretariat at ista.office@ista.ch.

Please send comments, suggestions or reports of problems relating to this method to the ISTA Seed Health Committee, c/o ISTA Secretariat.

Disclaimer

Whilst ISTA has taken care to ensure the accuracy of the methods and information described in this method description, ISTA shall not be liable for any loss or damage, etc. resulting from the use of this method.

Safety precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during weighing out of ingredients. It is assumed that persons carrying out this test are in a laboratory suitable for carrying out microbiological procedures and familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic techniques. Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Note on the use of the translations

The electronic version of the International Rules for Seed Testing includes the English, French, German and Spanish versions. If there are any questions on interpretation of the ISTA Rules, the English version is the definitive version.

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7-023: Detection of *Pseudomonas savastanoi* pv. *phaseolicola* in *Phaseolus vulgaris* (bean) seed

Host: *Phaseolus vulgaris* L.
Pathogen(s): *Pseudomonas savastanoi* pv. *phaseolicola* (*Psp*) (Burkh.) Gardan, Bollet, Abu, Ghorrah, Grimont & Grimont, syn. *P. syringae* pv. *phaseolicola* (Burkh.) Young, Dye & Wilkie

Prepared by: International Seed Health Initiative for Vegetable Crops, ISF (ISHI-Veg)

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Revision history

Version 1.0, 2007-01-01
Version 1.1, 2013-01-01: Definition of sample size
Version 1.2, 2014-01-01: Common name of host added
Version 1.3, 2017-01-01: Materials: numbers of Petri dishes for media deleted; Reporting results revised
Version 1.4, 2021-01-01: Sample size revised
Version 1.5, 2024-01-01: Sample size, Methods and General methods revised
Version 1.6, 2025-01-01: Background revised

Background

ISTA has published two working sheets (Nos. 65 and 66) for *Pseudomonas savastanoi* pv. *phaseolicola* (*Psp*) (Van Vuurde & Van den Bovenkamp, 1987; Jansing & Rudolph, 1996). Not only do the extraction methods in these working sheets differ, they also differ from the stationary overnight soaking commonly used in the testing laboratories of the seed industry. In practice, this has proven to be adequate in terms of sensitivity. Therefore, stationary overnight soaking is part of the present version for the detection of *Psp* and was incorporated in the validation study (Kurowski & Remeeus, 2007).

Both ISTA working sheets are based on dilution plating, although an immunofluorescence (IF) prescreening is part of working sheet No. 65. The present version (Kurowski & Remeeus, 2007) abandons this IF prescreening. Instead of plating in triplicate on modified sucrose peptone (MSP), as in working sheet No. 66, two plates of MSP and two plates of an additional medium, milk Tween™ (MT; Goszczynska & Serfontein, 1998), are used. MT can complement MSP, and has the advantage of being able to detect *Xanthomonas phaseoli* pv. *phaseoli* (*Xpp*) (previously *X. axonopodis* pv. *phaseoli* [*Xap*]) and *X. citri* pv. *fuscans* (*Xcf*) (previously *X. axonopodis* pv. *phaseoli* var. *fuscans* [*Xff*]), which is not possible on MSP. Furthermore, MT allows *Psp* to be distinguished from colonies of *Pseudomonas syringae* pv. *syringae*. The value of MT for detection of *Psp*, *Xpp* and *Xcf* has been demonstrated in practice and in method validation studies for both pathogens (Kurowski & Remeeus, 2007; Sheppard & Remeeus, 2006).

In the present version, the final identification of *Psp* is based on a pathogenicity assay on bean seedlings, as in working sheet No. 65; the phaseolotoxin assay of working sheet No. 66 is not widely used and does not identify all strains of the pathogen (Rico *et al.*, 2003). A subculturing step, to further assist in identifying suspect colonies, has been added to the method.

Treated seed

This method has not been validated for the determination of *Psp* on treated seed. Seed treatments may affect the performance of the method. (Definition of treatment: any process, physical, biological or chemical, to which a seed lot is subjected, including seed coatings. See 7.2.3.)

Sample size

The sample size (total number of seeds to be tested) and subsample size depend on intended use, the maximum acceptable infection level and the analytical sensitivity of the method. The minimum recommended working sample size is 5000 seeds and the maximum subsample size must be 1000 seeds.

Materials

Reference material: a known strain of *Pseudomonas savastanoi* pv. *phaseolicola* (*Psp*) or standardised reference material

Plates of MT medium: 90 mm Petri dishes

Plates of MSP medium: 90 mm Petri dishes

Plates of King's B (KB) medium: for subculturing (at least 1 sectored plate per subsample)

Polythene bags or containers: with sterile saline (0.85 % NaCl) plus Tween™ 20 (0.02 %; 0.2 ml/l) for soaking of seeds (volume [ml] required is equivalent to 2.5 × TSW [g])

Dilution bottles: containing 4.5 ml of sterile saline (2 per subsample). Other volumes may be acceptable (see General methods)

70 % ethanol or equivalent disinfecting product: for disinfection of surfaces and equipment

Incubator: capable of operating at 28 ±2 °C, 20–25 °C and 18–20 °C

Balance: capable of weighing to the nearest 0.001 g

pH meter: capable of being read to the nearest 0.01 pH unit

Automatic pipettes: check accuracy and precision regularly

Bean seedlings: susceptible to all races of the pathogen for pathogenicity test (e.g. 'Helda')

Cold room or refrigerator: operating at 5 ±4 °C

Sample preparation

This can be done in advance of the assay.

It is vital to exclude any possibility of cross-contamination between seed samples. It is therefore essential to disinfect all surfaces, containers, hands, etc. both before and after handling each sample. This can be achieved by using swabbing or spraying equipment with gloves and 70 % ethanol.

If the submitted sample is received in several packets, these should be combined by emptying into a new, clean polythene bag and mixing by hand to give a composite sample.

1. Count the number of seeds in a known weight. Estimate the thousand-seed weight (TSW) as:
   TSW = (weight of seeds / number of seeds) × 1000
2. Based on the estimated TSW, weigh out subsamples of the required size into new, clean polythene bags or containers.

Methods

Critical control points are indicated by CCP.

1. Extraction

1.1 Suspend seeds in sterile saline plus Tween™ 20 (0.02 % v/v) in a polythene bag or container. The volume of saline required in millilitres should be equivalent to 2.5 × TSW (g). For example: if TSW = 300 g, the volume of saline required is 2.5 × 300 = 750 ml (Olivier & Remeeus, 2004).

1.2 Soak subsamples overnight (16–18 h) at 5 ±4 °C).

2. Dilution and plating

2.1 Shake the polythene bag or container to obtain a homogenous extract before dilution.

2.2 Prepare a tenfold dilution series from the seed extract. Pipette 0.5 ml of the extract into 4.5 ml of sterile saline and vortex to mix (10^{-1} dilution). Pipette 0.5 ml of the 10^{-1} dilution into another 4.5 ml of sterile saline and vortex to mix (10^{-2} dilution)(see General methods).

2.3 Pipette 100 µl of each dilution and the undiluted seed extract onto plates of each of the selective media (MT, MSP) and spread over the surface with a sterile bent glass rod (see General methods).

2.4 Incubate inverted plates at 28 ±2 °C) and examine after 4–5 d (see section 5).

3. Positive control (culture or reference material)

3.1 Prepare a suspension of a known strain of *Psp* in sterile saline or reconstitute standardised reference material according to the supplier's instructions.

3.2 Dilute the suspension sufficiently to obtain dilutions containing approximately 10^{-2} to 10^{-4} colony-forming units (cfu) per millilitre. This may require up to seven tenfold dilutions from a turbid suspension.

3.3 Pipette 100 µl of appropriate countable dilutions onto plates of each of the selective media (MT, MSP) and spread over the surface with a sterile bent glass rod.

3.4 Incubate plates with the sample plates.

4. Sterility check

4.1 Plate a dilution series from a sample of the extraction medium (i.e. saline + Tween™ 20), containing no seeds, and plate on each of the media as for samples.

5. Examination of the plates
5.1 Examine sterility check and recovery of positive controls on semi-selective medium (CCP).
5.2 Examine the sample plates for the presence of typical *Psp* colonies by comparison with the positive control plates.
5.3 After 4–5 d on MT, *Psp* colonies are creamy white, flat, circular, 4.5–5 mm in diameter (Fig. 1).
5.4 After 4–5 d on MSP, *Psp* colonies are circular, raised globose, glistening and light yellow with a less dense centre. The medium around the colony turns light yellow after 3 d (Fig. 2).
5.5 The colony size and colour can differ within a sample.
5.6 Record the presence of suspect colonies (see General methods). If necessary, estimate the number of cfu of suspect and other colonies.
6. Confirmation and identification of suspect colonies
6.1 Subculture suspect colonies to sectored plates of KB. To prevent cross-contamination of isolates, use a new sectored plate for each subsample. The precise numbers of colonies subcultured will depend on the number and variability of suspect colonies on the plate; if present, at least six colonies should be subcultured per subsample (CCP).
6.2 Subculture the positive control isolate to a sectored plate for comparison.
6.3 Incubate sectored plates for 2–4 d at 28 ±2 °C).
6.4 Compare appearance of growth with positive control. On KB in general, *Psp* develops creamy or white circular and flat colonies (CCP)(Fig. 3).
6.5 Confirm the identity of isolates by pathogenicity on bean seedlings of known susceptibility (CCP).
6.6 Record results for each colony subcultured.
7. Pathogenicity assay
(Fenwick & Guthrie, 1969; Van Vuurde & Van den Bovenkamp, 1989)
7.1 Incubate seeds of a bean cultivar known to be susceptible to all races of *Psp* (e.g. 'Helda') in rolled germination paper for 3–4 d (crook-neck stage) at 18–20 °C in darkness.
7.2 Dip a sterile toothpick or needle in the bacterial culture on a 2–4 d KB culture (e.g. sectored plate).
7.3 Stab the needle through the cotyledon. Turn the toothpick or needle slightly while withdrawing to release bacteria. Re-infecting the toothpick or needle between inoculations is recommended.
7.4 Inoculate 2 seedlings per isolate.
7.5 Inoculate seedlings with the positive control isolate and a negative control with only a toothpick or needle (CCP).
7.6 Inoculated seedlings are transferred to damp soil in a humidity chamber (70–80 % RH) for 4–5 d at 20–25 °C (light:dark 12:12).
7.7 Record symptoms after 4–5 d and again at 8–10 d if necessary. After 4–5 d but before deterioration of the cotyledons, the flat inner sides of the cotyledons are inspected for typical 'greasy' spots at the point of inoculation (Fig. 4). Compare with positive control (CCP).

General methods

Preparation of tenfold dilution series: Each dilution should be prepared by pipetting 0.5 ml (±5 %) from a well-mixed seed extract or previous dilution into a universal bottle (screw-capped) or similar container containing 4.5 ml (±2 %) of sterile diluent and then vortexing to mix prior to the next dilution step. A new sterile pipette tip should be used for each dilution step. Pipettes should be checked regularly for accuracy and precision and recalibrated as necessary. It is acceptable to prepare tenfold dilutions using other volumes, provided that the laboratory can demonstrate that the required accuracy and precision can be achieved.

Plating of dilutions: This should be done as soon as possible after dilutions have been prepared, and certainly within 30 min. Working from the highest (most dilute) dilution to the undiluted extract, 0.1 ml is pipetted onto the centre of a surface-dry, labelled agar plate. The liquid should then be spread evenly over the entire surface of the medium with a bent glass rod. If care is taken to work from the highest to the lowest dilution (or undiluted extract) a single pipette tip and a single bent glass rod can be used for each sample. Ensure that all liquid has been absorbed by the agar before inverting and incubating plates. If necessary, allow plates to dry under a sterile airflow in a microbiological safety cabinet or laminar-flow hood.

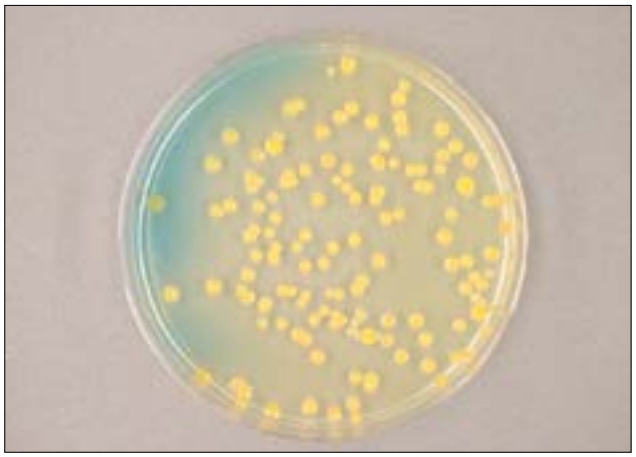

Figure 1. *Pseudomonas savastanoi* pv. *phaseolicola* colonies on MSP plates after 4 d are circular, raised globose, glistening and light yellow, and the medium around the colony turns light yellow.

Figure 2. *Pseudomonas savastanoi* pv. *phaseolicola* colonies on MT plates after 4 d are creamy white, flat, circular and 4.5–5 mm in diameter.

Figure 3. Isolation of *Pseudomonas savastanoi* pv. *phaseolicola* by sectoring on KB medium showing white, creamy and flat colonies.

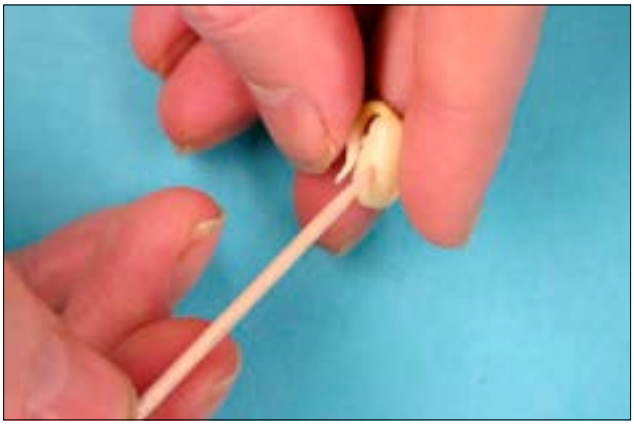

Figure 4. Germinated bean seedlings. The method of inoculation.

Figure 5a, b. Typical *Pseudomonas savastanoi* pv. *phaseolicola* symptoms in a pathogenicity test, indicated as a typical 'greasy' spot (arrows) at the point of inoculation.

Recording of dilution plates: Record the results for all dilution plates. The most accurate estimate of bacterial numbers should be obtained from spread plates with total number between 30 and 300 colonies. However this may be further complicated depending on the relative numbers of suspect pathogen and other colonies. In order to minimise effort, start recording with the highest dilution (most dilute) and count the number of suspect and the number of other colonies. If the total number of colonies on a plate greatly exceeds 300 there is little value in trying to make a precise count if a more reliable count has already been obtained from a more dilute plate, in which case it is sufficient to record the number of colonies as 'm' (many) if they are still separate or 'c' (confluent) if they have run together.

Sectored plates: Using a laboratory marker pen, draw lines on the base of a standard 90 mm plate (Petri dish) to divide it into six equal sectors. Subculture single colonies from dilution plates and make a single zigzagged streak within a single sector on the plate. Take care to leave sufficient space between each isolate to ensure the growth does not coalesce. Thus six suspect colonies can be subcultured to each sectored plate. Separate plates should be used for each sample/subsample. If the purity of subcultured isolates is doubtful, they should be further streaked out on whole plates.

Reporting results: The result of a seed health test should indicate the scientific name of the pathogen detected and the test method used. When reported on an ISTA Certificate, results are entered under 'Other Determinations'.

The report must indicate the number of seeds tested.

In the case of a negative result (pathogen not detected in any subsample), the results must be reported as 'not detected'.

In the case of a positive result, the report must indicate the number of positive subsamples out of the total number tested. The number of cfu can be indicated.

Quality assurance

A record should be kept of the date and results of pipette calibration checks.

It is essential that operators have received appropriate training and use automatic pipettes correctly.

Critical control points (CCP)

- Dilution plates prepared from positive control isolate(s) or reference material should give single colonies with typical morphology (Step 5.1).
- The numbers of colonies on dilution plates prepared from the positive control isolate(s) or reference material should be similar on both media (Step 5.1).
- Numbers of bacteria on dilution plates should be consistent with the dilution (i.e. should decrease approximately tenfold with each dilution)(Step 5.1).
- There should be no growth on dilution plates prepared as a sterility check (Step 5.1).
- Due to the potential for non-pathogenic isolates to be present in seed lots together with pathogenic isolates, it is essential to subculture (Step 6.1), if present, at least the minimum number of suspect colonies specified (six per subsample). Compare the appearance of growth of subcultured isolates with that of positive controls (Step 6.4). and test all *Pseudomonas*-like subcultured isolates for pathogenicity (Step 6.5).
- Positive control isolates should be included in every pathogenicity test (Step 7.5).
- The positive control isolate should give typical symptoms in pathogenicity test (Step 7.7).
- The activity units per gram of some antibiotics may vary between batches. It may be necessary to adjust the weight or volume added to ensure that the final number of units per litre of medium is consistent (MT and MSP media).

Media and solutions

Sterile saline

(van Vuurde *et al.*, 1989)

Sodium chloride (NaCl): 8.5 g/l
Distilled/deionised water: 1000 ml

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add 1000 ml of distilled/deionised water.
3. Dissolve and dispense into final containers.
4. Autoclave at 121 °C and 15 psi for 15 min.
5. For extraction of seeds, add 0.2 ml of sterile Tween™ 20 per litre after autoclaving.

Storage

Provided containers are tightly closed, sterile saline may be stored for several months before use.

Modified sucrose peptone medium

(Mohan & Schaad, 1987)

Sucrose: 20.0 g
Proteose peptone No. 3: 5.0 g
K_2HPO_4: 0.5 g
$MgSO_4 \cdot 7H_2O$: 0.25 g
Agar: 20.0 g
Distilled/deionised water: 1000 ml
Cycloheximide [a]: 200.0 mg
Cephalexin [b]: 80.0 mg
Vancomycin [c]: 10.0 mg
Bromothymol blue (15 mg/ml 95 % ethanol) [d]: 15.0 mg

[a–d] Added after autoclaving. Antibiotic amounts for guidance only (CCP).

[a] Dissolve 2 gcycloheximide in 10 ml 70 % ethanol. Add 1 ml/l.

[b] Dissolve 800 mg cephalexin in 10 ml 70 % ethanol. Add 1 ml/l.

[c] Dissolve 100 mg vancomycin in 10 ml 70 % ethanol. Add 1 ml/l.

[d] Dissolve 150 mg bromothymol blue in 10 ml 95 % ethanol. Add 1 ml/l.

Filter sterilise when antibiotics are dissolved in water rather than 70 % ethanol.

Note: Nystatin can be used as an alternative for cycloheximide to control fungi. Dissolve 400 mg nystatin in 10 ml 70 % ethanol; add 1 ml to cool medium.

Preparation

1. Weigh out all ingredients except antibiotics and bromothymol blue into a suitable container.
2. Add 1000 ml of distilled/deionised water.
3. Steam to dissolve.
4. Autoclave at 121 °C and 15 psi for 15 min.
5. Prepare antibiotic and bromothymol blue solutions.
6. Allow medium to cool to approximately 50 °C before adding antibiotic and bromothymol blue solutions.
7. Mix the molten medium gently to avoid air bubbles and pour plates (22 ml per 90 mm plate).
8. Leave plates to dry in a laminar flow bench or similar before use.

Storage

Store prepared plates inverted in polythene bags at 4–8 °C and use within 4 weeks of preparation to ensure activity of antibiotics.

Milk Tween™ medium

(adapted from Goszczynska & Serfontein, 1998)

Section A
Proteose peptone No. 3: 10.0 g/l
$CaCl_2$: 0.25 g/l
Tyrosine: 0.5 g/l
Agar: 15.0 g/l
Distilled/deionised water. 500 ml

Section B
Skim milk powder (Oxoid, Sigma; CCP): 10.0 g/l
Distilled/deionised water: 500 ml

Section C
Tween™ 80: 10.0 ml

Section D
Nystatin [a]: 40 mg (1 ml)
Cephalexin [b]: 80 mg (1 ml)
Vancomycin [c]: 10 mg (1 ml)

[a–c] Added after autoclaving. Antibiotic amounts for guidance only (CCP).
[a] Dissolve 400 mg nystatin in 10 ml 70 % ethanol.
[b] Dissolve 800 mg cephalexin in 10 ml 70 % ethanol.
[c] Dissolve 100 mg vancomycin in 10 ml 70 % ethanol.

Filter sterilise when antibiotics are dissolved in water rather than 70 % ethanol.

Preparation

1. Weigh out all ingredients in section A into a suitable container.
2. Add 500 ml of distilled/deionised water.
3. Dissolve ingredients.
4. In a separate container, dissolve skim milk powder in 500 ml distilled/deionised water.
5. Separately prepare 10 ml Tween™ 80.
6. Sterilise preparations from section A, skim milk solution (section B) and Tween™ 80 (section C) separately at 121 °C and 15 psi for 15 min.
7. After sterilisation of all components, aseptically add sterilised skim milk preparation and sterilised Tween™ 80 to sterilised ingredients in section A.
8. Prepare antibiotic solutions (section D).
9. Allow medium to cool to approximately 50 °C and add antibiotics.
10. Mix gently to avoid air bubbles, and pour 22 ml of mixture onto each 90 mm plate.
11. Leave plates to dry on a laminar flow bench or similar before use.

King's B (KB) medium

(King *et al.*, 1954)

Proteose peptone No. 3: 20.0 g/l
K_2HPO_4: 1.5 g/l
$MgSO_4 \cdot 7H_2O$: 1.5 g/l
Glycerol: 15.0 ml
Agar: 15.0 g/l
Distilled/deionised water: 1000 ml

Preparation

1. Weigh out all ingredients into a suitable oversize container (i.e. 250 ml of medium in a 500 ml bottle or flask) to allow swirling of medium just before pouring.
2. Add 1000 ml of distilled/deionised water.
3. Steam to dissolve.
4. Autoclave at 121 °C and 15 psi for 15 min.
5. Allow medium to cool to approximately 50 °C.
6. Pour plates (22 ml per 90 mm plate).
7. Leave plates to dry in a laminar flow bench or similar before use.

Storage

Store prepared plates inverted in polythene bags at 4–8 °C. Prepared plates can be stored for several months provided they do not dry out.

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