International Rules for Seed Testing
Annexe to Chapter 7: Seed Health Testing Methods

7-023: Detection of *Pseudomonas savastanoi* pv. *phaseolicola* on *Phaseolus vulgaris*

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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, damage etc. resulting from the use of this method.

Crop: *Phaseolus vulgaris* (bean)

Pathogen: *Pseudomonas savastanoi* pv. *phaseolicola* syn. *Pseudomonas syringae* pv. *phaseolicola* (Halo Blight) (Gardan et al., 1992)

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Background

ISTA has published two working sheets (Nos. 65 and 66) for *Pseudomonas savastanoi* pv. *phaseolicola* (Psp; Van Vuurde and Van den Bovenkamp, 1987; Jansing and 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 and 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 and 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 and Serfontein, 1998), are used. MT can complement MSP, and has the advantage of being able to detect *Xanthomonas axonopodis* pv. *phaseoli* (Xap), which is not possible on MSP. Furthermore, MT allows *Pseudomonas savastanoi* pv. *phaseolicola* to be distinguished from colonies of *Pseudomonas syringae* pv. *syringae*. The value of MT for detection of Psp and Xap has been demonstrated in practice and in method validation studies for both pathogens (Kurowski and Remeeus, 2007; Sheppard and 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.

Validation studies

Kurowski, C. and Remeeus, P.M. (2007)
Copies are available: by E-mail from ista.office@ista.ch; by mail from the ISTA Secretariat.

Safety precautions

Ensure you are familiar with hazard data and take appropriate safety precautions, especially during preparation of media, autoclaving, and weighing out of ingredients. It is assumed that this procedure is being carried out in a microbiological laboratory by persons familiar with the principles of Good Laboratory Practice, Good Microbiological Practice, and aseptic technique. Dispose of all waste materials in an appropriate way (e.g. autoclaving or disinfection) and in accordance with local health, environmental and safety regulations.

Treated seed

This method has not been validated for the determination of *Pseudomonas savastanoi* pv. *phaseolicola* (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 and subsample size

The sample (total number of seeds tested) or subsample size to be tested depends on the desired tolerance standard (maximum acceptable percentage of seeds infested) and detection limit (theoretical minimum number of pathogen propagules per seed which can be detected). In any case, the subsample size should not exceed 1000 seeds.

Materials

Reference material: a known strain of *Pseudomonas savastanoi* pv. *phaseolicola* or standardized reference material.

Plates of MT medium: 9.0 cm Petri dishes (3 plates of each medium per subsample + controls).

Plates of MSP medium: 9.0 cm Petri dishes (3 plates of each medium per subsample + controls).

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. cv. Helda).
Cold room or refrigerator: operating at 5 ± 4 °C).

Sample preparation

This can be done in advance of the assay.

1. 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.
2. 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.
3. Count the number of seeds in a known weight. Estimate the thousand-seed weight (TSW) as:

   TSW = (weight of seeds/number of seeds) × 1000

4. 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 and 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 stand-

ardized reference material according to the supplier's instructions.

3.2 Dilute the suspension sufficiently to obtain dilutions containing approximately 10^2 to 10^4 cfu/mL. 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 Estimate the number of suspect and other colonies (see General methods).

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 and Guthrie, 1969; Van Vuurde and Van den Bovenkamp, 1989)

7.1 Incubate seeds of a bean cultivar known to be susceptible to all races of Psp (e.g. cv. 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-infesting 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

(common to many test procedures)

1. 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.

2. 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.

3. 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 minimize 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 suf-

ficient to record the number of colonies as 'm' (many) if they are still separate or 'c' (confluent) if they have run together.

4. **Sectored plates**
   Using a laboratory marker pen draw lines on the base of a standard 9 cm 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.
5. **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 International Seed Analysis Certificate*, results are entered under Other Determinations.
   In the case of a negative result (pathogen not detected in any subsamples), the results should be reported in terms of the tolerance standard and detection limit. The tolerance standard depends on the total number of seeds tested, n, and is approximately 3/n (P=0.95) (see Roberts et al., 1993); the detection limit per subsample is equal to the detection limit per mL multiplied by the volume of extract.
   In the case of a positive result, the report should indicate the mean number of pathogen propagules (cfu) per seed and either the number of positive subsamples out of the total number tested and the sample size or the maximum likelihood estimate of the proportion of infested seeds.

Quality assurance

General

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

(identified in the methods by "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).

Preparation of sterile saline

(van Vuurde et al., 1989)
Sodium chloride (NaCl): 8.5 g/L.
Distilled/de-ionized water: 1000 mL.

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add 1000 mL of distilled/de-ionized water.
3. Dissolve and dispense into final containers.
4. Autoclave at 121 °C and 15 p.s.i. 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.

Preparation of MSP agar medium

(Mohan and 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/de-ionized water: 1000 mL.
Cycloheximide*: 200.0 mg.
Cephalexin*: 80.0 mg.
Vancomycin*: 10.0 mg.
Bromothymol blue (15 mg per mL 95% EtOH)*: 15.0 mg.
* Added after autoclaving

Preparation

1. Weigh out all ingredients except antibiotics and bromothymol blue into a suitable container.
2. Add 1000 mL of distilled/de-ionized water.
3. Steam to dissolve.
4. Autoclave at 121 °C and 15 p.s.i. 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 9.0 cm plate).
8. Leave plates to dry in a laminar flow bench or similar before use.

Antibiotics (amounts for guidance only; CCP)

a) Dissolve 2 g cycloheximide 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 sterilize 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.

Storage

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

Preparation of MT agar medium

(adapted from Goszczynska and Serfontein, 1998)

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/de-ionized water. 500 mL.

B **Skim milk powder (Oxoid, Sigma; CCP):** 10.0 g/L.
Distilled/de-ionized water: 500 mL.

C **Tween 80:** 10.0 mL.

D **Nystatin*:** 40 mg (1 mL).
Cephalexin*: 80 mg (1 mL).
Vancomycin*: 10 mg (1 mL).
* Added after autoclaving

Preparation

1. Weigh out all ingredients in section A into a suitable container.
2. Add 500 mL of distilled/de-ionized water.
3. Dissolve ingredients.
4. In a separate container, dissolve skim milk powder in 500 mL distilled water.
5. Separately prepare 10 mL Tween 80.
6. Sterilize preparations from section A, skim milk solution (section B) and Tween 80 (section C) separately at 121 ºC and 15 p.s.i. for 15 min.
7. After sterilization of all components, aseptically add sterilized skim milk preparation

and sterilized Tween 80 to sterilized ingredients in section A.
9. Prepare antibiotic solutions (section D).
10. Allow medium to cool to approximately 50 ºC and add antibiotics.
11. Mix gently to avoid air bubbles, and pour 22 mL of mixture onto each 9.0 cm plate.
12. Leave plates to dry on a laminar flow bench or similar before use.

Antibiotics (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 sterilize when antibiotics are dissolved in water rather than 70% ethanol.

Preparation of King's B (KB) agar 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/de-ionized 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/de-ionized water.
3. Steam to dissolve.
4. Autoclave at 121 °C and 15 p.s.i. for 15 min.
5. Allow medium to cool to approximately 50 °C.
6. Pour plates (22 mL per 9.0 cm 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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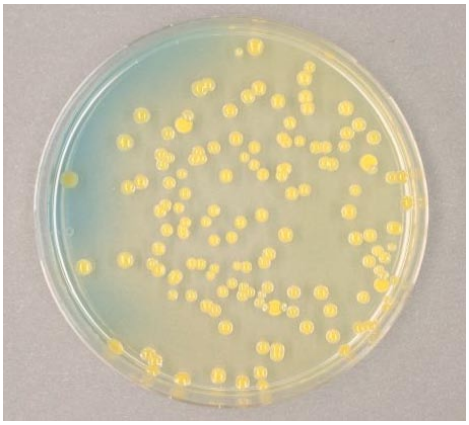


Fig. 1. *Pseudomonas savastanoi* pv. *phaseolicola* colonies on MSP plates after 4 d are circular, raised globose, glistening and light yellow, and the medium around the colony turns light yellow.

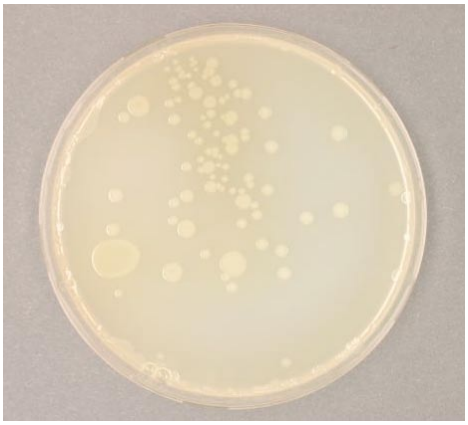
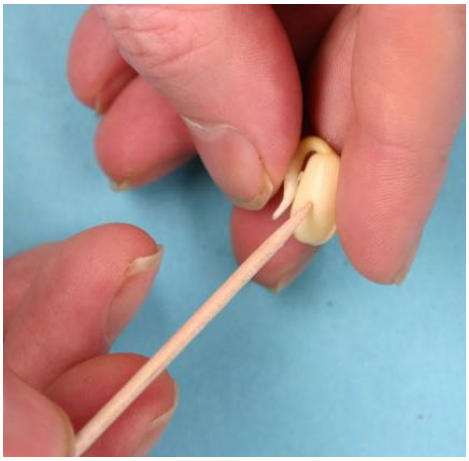
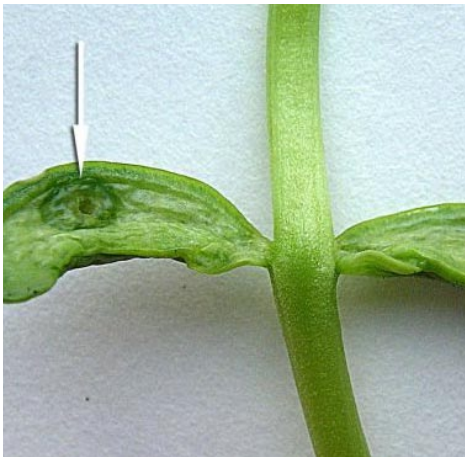


Fig. 2. *Pseudomonas savastanoi* pv. *phaseolicola* colonies on MT plates after 4 d are creamy white, flat, circular and 4.5–5 mm in diameter.

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

A





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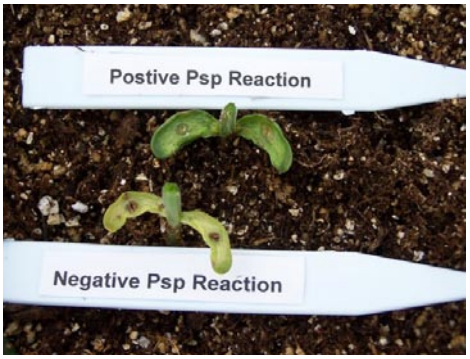

C

Fig. 4. Germinated bean seedlings. **A** The method of inoculation. **B** Typical *Pseudomonas savastanoi* pv. *phaseolicola* symptoms in a pathogenicity test, indicated as a typical 'greasy' spot (**C**) at the point of inoculation.