



HAL
open science

Detection of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* on *Phaseolus vulgaris* (bean)

Valérie Grimault, Valérie Olivier, M. Rolland, Armelle Darrasse, Marie-Agnès
Jacques

► To cite this version:

Valérie Grimault, Valérie Olivier, M. Rolland, Armelle Darrasse, Marie-Agnès Jacques. Detection of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* on *Phaseolus vulgaris* (bean). International Rules for Seed Testing Annexe to Chapter 7: Seed Health Testing Methods, 2014, International Rules for Seed Testing, 7-021, pp.7-021-1-20. hal-01134886

HAL Id: hal-01134886

<https://hal.science/hal-01134886>

Submitted on 24 Mar 2015

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



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



7-021: Detection of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis*  *phaseoli* var. *fuscans* on *Phaseolus vulgaris* (bean)

Important notice

The pathogenicity test for this method is under review due to the occurrence of false positive results. Please see the ISTA website for more details.

Published by: International Seed Testing Association (ISTA), Bassersdorf, Switzerland
2014

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: *Xanthomonas axonopodis* pv. *phaseoli* (common blight) and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans*

Authors: Grimault V.¹, Olivier V.², Rolland M.³, Darrasse A.⁴ and Jacques M. A.⁴

¹GEVES-SNES, rue Georges Morel, B.P. 90024, 49071 Beaucouzé CEDEX, France
E-mail: valerie.grimault@geves.fr

²ANSES-Plant health laboratory, 7 rue Jean Dixmèras, 49044 Angers CEDEX 01, France
E-mail: valerie.olivier@anses.fr

³BioGEVES, rue Georges Morel, B.P. 90024, 49071 Beaucouzé CEDEX, France
E-mail: mathieu.rolland@geves.fr

⁴INRA, UMR1345 IRHS, SFR 149 Quasav, Bâtiment C, 42, rue Georges Morel, BP 60057, F-49071 Beaucouzé CEDEX, France
E-mail: Marie-Agnes.Jacques@angers.inra.fr

Prepared by: ISTA and International Seed Health Initiative for Vegetables, ISF (ISHI-Veg)

Revision history:

Version 1.0, 2006-02-22

Version 2.0, 2006-08-18

Version 2.1, 2010-01-01: Editorial change to Critical control points (p. 7-021-9)

Version 2.2, 2012-01-01: Notice regarding review of pathogenicity test

Version 2.3, 2013-01-01: Definition of sample size

Version 2.4, 2014-01-01: Common name of host added

Version 3.0, 2014-01-01

Background

This method is derived from the validation studies carried out by ISTA in 2003, in collaboration with the International Seed Health Initiative for Vegetables (ISHI-Veg) (Sheppard and Remeus, 2005). For routine testing of bean seed a combination of two complementary semi-selective media, MT and XCP1, is recommended with a pathogenicity test to confirm suspect isolates.

The two media, XCP1 and MT, have been chosen for their ease of use and selectivity for *X. axonopodis* pv. *phaseoli*. In addition both media can be used to detect both *X. axonopodis* pv. *phaseoli* and *X. axonopodis* pv. *phaseoli* var. *fuscans*. Although initially the morphology of fuscans and non-fuscans strains of *X. axonopodis* pv. *phaseoli* appear to be similar on the media after a longer incubation the fuscans colonies are distinguished by a distinct brown pigmentation. A further advantage of MT medium is that it can also be used for identifying other bacterial seed-borne pathogens of beans, e.g. *Pseudomonas savastanoi* pv. *phaseolicola* and *Pseudomonas syringae* pv. *syringae*.

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

In 2010 in the USA and France conflicting data were obtained with the new ISTA method. Research in France (GEVES and INRA) and in the Netherlands (Naktuinbouw) showed that some isolates that were responsible for positive results were causing symptoms in the pathogenicity assay but were not identified as Xap based on molecular methods (genetic bacterial fingerprinting in the Netherlands and pathogen specific PCR's in France). Therefore it was concluded that the pathogenicity assay used in the ISTA method, a crucial step in the Xap test, is not reliable enough.

A new pathogenicity assay was developed at INRA to allow a reliable characterization of the aggressiveness of *X. axonopodis* pv. *phaseoli* wild type strains and mutants (Darsonval *et al.*, 2009). A comparison study of the new pathogenicity test and primers specific for *X. axonopodis* pv. *phaseoli* fuscans and non fuscans isolates (Audy *et al.*, 1994; Boureau *et al.*, 2012) was carried out as a collaboration between ISTA, ANSES, INRA and ISHI-Veg. This study showed that the new pathogenicity test and Audy *et al.*, (1994) primers were good confirmation tools and that Diaggene (Boureau *et al.*, 2012) primers gave good results but their use did not improve sensitivity of the method.

Two options are proposed for confirmation of suspect isolates:

- Option 1:** Pathogenicity assay, for laboratories not equipped or experienced with PCR. In this case, CCP must be followed and target and non target controls added (*X. vesicatoria*, Xap, water). This option is also valuable and less time consuming when few suspect isolates have been detected but requires a growth chamber or greenhouse equipped for high relative humidity (RH).
- Option 2:** PCR test with Audy *et al.*, (1994) primers. This option can be used for laboratories experienced and equipped for PCR, when a short delay is needed for obtaining results and/or a high number of suspect isolates have been detected.

Validation studies

Grimault V., Olivier V., Rolland M., Darrasse A. and Jacques M.A. (2012).

Copies are available: by E-mail from ista.office@ista.ch; by mail from the ISTA Secretariat, Zürichstrasse 50, 8303 Bassersdorf, Switzerland.

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. autoclave, disinfect) and in accordance with local health, environmental and safety regulations.

Ethidium bromide

Ethidium bromide is carcinogenic. Use ethidium bromide according to safety instructions. It is recommended to manipulate solution instead of powder. Some considerations are mentioned below.

- Consult the Material Safety Data Sheet on ethidium bromide before using the chemical.
- Always wear personal protective equipment when handling ethidium bromide. This includes wearing a lab coat, nitrile gloves and closed toe shoes.
- Leave lab coats, gloves, and other personal protective equipment in the lab once work is complete to prevent the spread of ethidium bromide or other chemicals outside the lab.
- All work with ethidium bromide is to be done in an "ethidium bromide" designated area in order to keep ethidium bromide contamination to a minimum.

UV light

UV light must not be used without appropriate precautions. Ensure that UV protective eye-wear is utilized when visualizing ethidium bromide.

Treated seed

Seed treatments may affect the performance of this test. It must only be performed on untreated seed.

Sample and subsample size

The sample (total number of seeds tested) and 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). The minimum recommended sample size is 5000 seeds. In any case the maximum subsample size should be 1000 seeds.

Materials

Reference material: a known strain of *fuscans* or non-*fuscans* types of *X. axonopodis* pv. *phaseoli* (positive control) and of *X. vesicatoria* (negative control) or standardized reference material

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

Plates of XCP1 medium: 9.0 cm Petri dishes (3 plates of each medium per subsample + controls)

Plates of YDC: for subculture (at least 1 per subsample)

Polythene bags or containers: of sterile saline (0.85 % NaCl) plus Tween 20 (0.02 % – 0.2 mL per litre) for soaking seeds (volume (mL) required is equivalent to $2.5 \times \text{TSW}$ (g))

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

70 % ethanol: for disinfection of surfaces, equipment

Incubator operating at 28 ± 2 °C

Balance: capable of weighing to the nearest 0.001 g

Automatic pipettes: check accuracy and precision regularly

Bean seedlings: susceptible to all races of the pathogen for pathogenicity test (e.g. Michelet, Contender)

Growth chamber: capable of operating at 28 °C or greenhouse, with high humidity (95 % RH), with quarantine status

Milli Q and chemicals: for PCR preparation

Sterile microtubes (1.5 mL; 0.2 mL)

Microliter pipettes: e.g. Gilson, Finn, with sterile filtered tips (1 µL–1000 µL)

Conventional thermocycler

Electrophoresis equipment: 1.5–2 % agarose gels

DNA visualizing system: BET or analogue reagent, UV imaging apparatus

PCR primers (Audy et al., 1994):

p7X4c: 5' ggcaacacccgatccctaacagg 3'

p7X4e: 5' cgccggaagcacgatcctcga ag 3'

Sample preparation

1. This can be done in advance of the assay.
2. 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 swabbing/spraying equipment and gloved hands with 70 % ethanol.
3. 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.
4. Count the number of seeds in a known weight. Estimate the Thousand Seed Weight (TSW) as:
$$\text{TSW} = (\text{weight of seeds} / \text{number of seeds}) \times 1000$$
5. Based on the estimated TSW, weigh out subsamples of the required size into new, clean polythene bags or containers.

Method

(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 mL should be equivalent to $2.5 \times \text{TSW (g)}$ e.g. TSW = 300 g, therefore volume of saline required is $2.5 \times 300 = 750$ mL (Olivier and Remeeus, 2004).
- 1.2. Soak sub samples overnight (16–18 h) at 5 °C (± 4 °C).

2. Dilution and plating

- 2.1. Shake containers or polythene bags 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 μ l of each dilution and the undiluted seed extract onto plates of each of the selective media (MT and XCP1) 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.

3. Positive control (culture or reference material)

- 3.1. Prepare a suspension of a known strain of *X. axonopodis* pv. *phaseoli*, fuscans and non-fuscans, in sterile saline or reconstitute standardised reference material according to the supplier's instructions.
- 3.2. Dilute suspension sufficiently to obtain dilutions containing approximately 10^2 to 10^4 cfu/mL. This may require up to seven ten-fold dilutions from a turbid suspension.
- 3.3. Pipette 100 μ l of appropriate countable dilutions onto plates of each of the selective media (MT and XCP1) and spread over the surface with a sterile bent glass rod.
- 3.4. Incubate plates with the sample plates.

4. Sterility check

- 4.1. Prepare a dilution series from a sample of the extraction medium (i.e., saline plus 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 control on semi-selective medium (**CCP**).
- 5.2. Examine the sample plates for the presence of typical *X. axonopodis* pv. *phaseoli* colonies by comparison with the positive control plates.
- 5.3. After 4–5 d on MT, *X. axonopodis* pv *phaseoli* colonies are yellow distinguished by two zones of hydrolysis; a large clear zone of casein hydrolysis and a smaller milky zone of Tween 80 lysis (Fig. 1a and b). The fuscans of *X. axonopodis* pv *phaseoli* colonies produce a brown diffusible pigment. If not visible after 4 d incubate for an additional day. Often the fuscans type colonies show Tween 80 lysis.
- 5.4. After 4–5 d on XCP1, *X. axonopodis* pv *phaseoli* colonies are yellow, glistening and surrounded by a clear zone of starch hydrolysis (Fig. 2b). The fuscans of *X. axonopodis* pv *phaseoli* colonies produce a brown diffusible pigment after 5 d of incubation (Fig 2a). Often the fuscans type colonies show Tween 80 lysis.

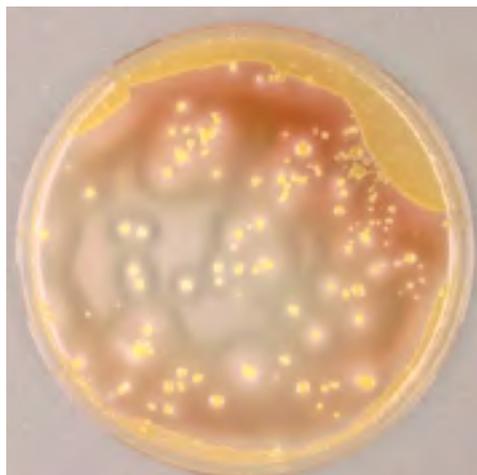
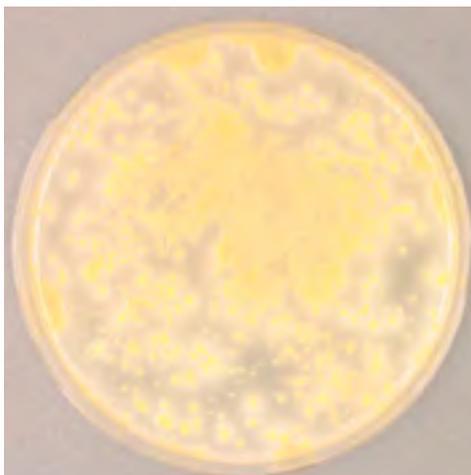
**Fig. 1a****Fig. 1b**

Fig. 1. *X. axonopodis* pv. *phaseoli* colonies on MT plates after 4 d indicated by a large clear zone of casein hydrolysis (a) and a smaller milky zone of Tween 80 lysis (b).

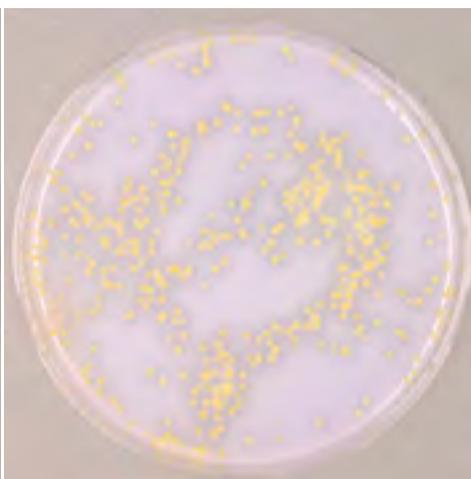
**Fig. 2a****Fig. 2b**

Fig. 2. *X. axonopodis* pv. *phaseoli* colonies, fuscans (a) and non-fuscans (b), on XCP1 plates showing a clear zone of starch hydrolysis and fuscans on XCP1 showing a milky zone, after 4 d

- 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/identification of suspect colonies*
 - 6.1. Subculture suspect colonies to sectoried plates of YDC. To avoid the potential for cross-contamination of isolates, use a new sectoried 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 (Fig. 3).
 - 6.3. Incubate sectored plates for 24–48 h at 28 ± 2 °C.
 - 6.4. Compare appearance of growth with positive control. On YDC *X. axonopodis* pv *phaseoli* colonies are yellow and mucoid in appearance (Fig. 3) (**CCP**).
 - 6.5. Confirm the identity of isolates by pathogenicity test on bean seedlings of known susceptibility (option 1) or by PCR (option 2).
 - 6.6. Record results for each colony subcultured.
7. **Pathogenicity (CCP)** (Darsonval *et al.*, 2009): Option 1
- 7.1. Grow seedlings of a bean cultivar known to be highly susceptible to Xap (e.g. Flavert or Michelet) at 20–30 °C in small pots until the first trifoliate leaf stage (approximately 16 days after sowing).
 - 7.2. Make a 10^7 cfu/mL (**CCP**) suspension in distilled/deionized water of a culture obtained after growth (24 or 48 h), 28 °C on YDC (i.e. sectored plate).
 - 7.3. Inoculation: dip first trifoliate leaf for 30 s in a container containing inoculum (beaker) (Fig. 4).
 - 7.4. The number of plants which should be inoculated is 3 plants per suspect isolate.
 - 7.5. Inoculate plants with one positive *X. axonopodis*, and 2 negative controls: *X. vesicatoria* and distilled/deionized water.
 - 7.6. Incubate at 28 °C, 16 h light, 95 % RH; 25 °C, 8 h dark, 95 % RH (**CCP**).
 - 7.7. Record symptoms from 5 to 11 days (depending on when symptoms begin) after inoculation. Compare with positive and negative controls. Typical Xap symptoms are water-soaked spots. Necrosis of lesions can develop and in case of very aggressive isolates lead to death of tissues (Fig. 5a, b, c, d). No lesions occur on negative controls (Fig. 6)
8. **Polymerase Chain Reaction (PCR)** (Audy *et al.* (1994): Option 2
- 8.1. Make a slightly turbid cell suspension at 10^7 cfu/mL (OD_{600nm} approximately 0.05) in 1.0 mL sterile distilled/deionized water from the suspect colonies cultured on YDC medium, and the positive and negative controls (**CCP**). Boil the suspension for 5 min at 95 °C for DNA extraction. Store at –20 °C until identification (**CCP**).
 - 8.2. Use the following *X. axonopodis* pv. *phaseoli* specific pair of primers from Audy *et al.* (1994) that will give a product of 800bp:
p7X4c: 5' ggcaacacccgatccctaaacagg 3'
p7X4e: 5' cgccggaagcac gat cctcgaag 3'
 - 8.3. Prepare the reaction mixture (page 7-021-■■■■, adapted by INRA) (**CCP**). Carry out the PCR reactions in 0.2 mL thin-walled PCR tubes in a final volume of 20 μ L (16 μ L reaction mixture + 4 μ L boiled bacterial suspension).
 - 8.4. PCR profile: An initial 3 min incubation at 94 °C followed by 35 cycles of 1 min at 94 °C, 2 min at 72 °C. A final 10 min incubation at 72 °C and infinity at 12 °C (**CCP**). During each amplification run, in addition to the positive and negative controls extracted in 8.1, a PCR negative control (DNA extract replaced by molecular biology grade water) is added.
 - 8.5. Fractionate 10 μ L of the PCR products and water (negative PCR control) by gel electrophoresis in a 1.5 % agarose gel in 1X Tris-acetate EDTA (TAE buffer) (**CCP**). Include a 100 bp ladder. Stain with ethidium bromide in a bath and rinse in water.

**Figure 3a****Figure 3b**

Figure 3. *X. axonopodis* pv. *phaseoli* colonies, fuscans (a) and non-fuscans (b), on YDC plates after 2 d are brown and yellow in appearance.



Figure 4. Inoculation by dipping first trifoliate leaf for 30 seconds in a beaker containing inoculum.



Figure 5. *Phaseolus vulgaris* leaves 5-11 days after inoculation with typical Xap water-soaked spots (a), necrosis (b, c) and dead tissues (d).

8.6. Analyse the amplification products for a *X. axonopodis* pv. *phaseoli* specific product of 800 bp (**CCP**) = positive identification of *X. axonopodis* pv. *phaseoli*, no band = negative identification (Fig. 7).

In case of a positive identification of *X. axonopodis* pv. *phaseoli*, as a low risk of false positive result is present (Audy et al. (1994) primers detect *X. axonopodis* pv. *dieffenbachiae* which are not supposed to be present on bean seeds), a pathogenicity test can be performed as complementary information.



Figure 6. *Phaseolus vulgaris* leaves 5–11 days after inoculation with a negative control.

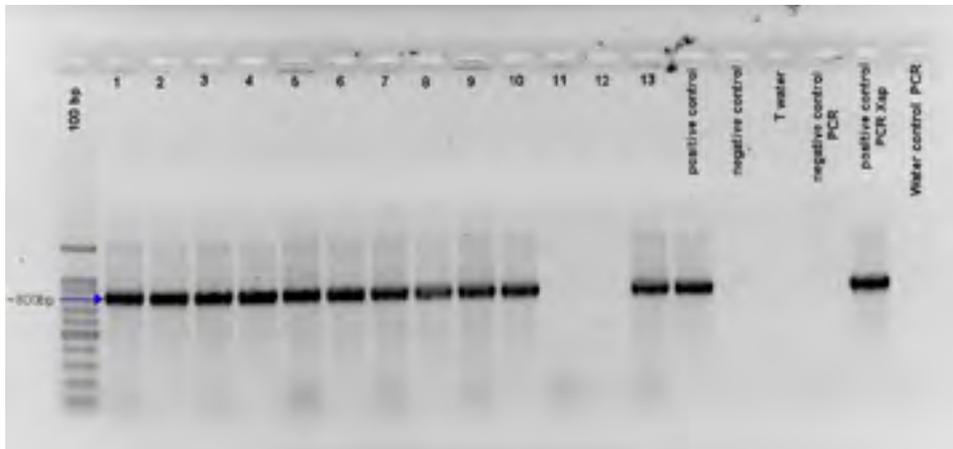


Figure 7. Agarose gel showing Xap specific band at 800 bp (Audy et al, (1994) primers)

General methods (common to many test procedures)

1. Preparation of ten-fold dilution series

Each dilution should be prepared by pipetting 0.5 mL ($\pm 5\%$) from a well-mixed seed extract or previous dilution into a universal bottle (screw-capped) or similar container containing 4.5 mL ($\pm 2\%$) 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 re-calibrated as necessary. It is acceptable to prepare ten-fold 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 air-flow 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 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.

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 and the test method used. When reported on an ISTA 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 (Most Probable Number).

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 by **CCP** in the methods)

- Dilution plates prepared from positive control isolate(s) or reference material, should give single colonies with typical morphology (Step 5.1 and 6.4).
- 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). Note that recovery of the fuscans type *X. axonopodis* pv. *phaseoli* is in general lower on MT than on XCP1.
- Numbers of bacteria on dilution plates should be consistent with the dilution (i.e. should decrease approximately ten-fold 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) and to test all *Xanthomonas*-like subcultured isolates for pathogenicity (Step 6.5).
- Positive control isolates should be included in every pathogenicity test (Step 7.6).
- The positive control isolate should give typical symptoms in a pathogenicity test (Step 7.8).
- The quality of milk powder is vital to develop the hydrolysis of milk in MT medium. Two milk sources that work well are Oxoid and Sigma brands.
- The activity (g) 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 XCP1 media).
- Due to the potential for non-pathogenic isolates to be present in seedlots together with pathogenic isolates, it is essential to subculture if present, at least the minimum number of suspect colonies specified (six per subsample) (Step 6.1), and to test all *Xanthomonas*-like subcultured isolates in pathogenicity or PCR test (Step 6.5).
- For pathogenicity assay, a concentration of 10^7 cfu/mL must be used as lower concentrations can lead to false negative results and higher concentrations to false positive results. The humidity during incubation must be very high (minimum 95%) to obtain water-soaked lesions. If the humidity is too low, necrotic lesions can develop without any visible water-soaked spot making more difficult the interpretation of the result.

- Positive and negative control isolates and negative PCR control should be included in every PCR test (Step 8.1).
- DNA extracted by boiling cannot be stored for a very long time. If stored at -20°C, positive and negative controls stored in the same conditions as suspect isolates' DNA must be used.
- The preparation of PCR mixture (Step 8.1, 8.4), and the preparation of agarose gel for electrophoresis (Step 8.5) should be adapted to available material and equipment of individual laboratories testing for of *X. axonopodis* pv. *phaseoli* under the condition that results will be validated on PCR controls.

Preparation of sterile saline

(van Vuurde *et al.*, 1989)

Compound	g/L
Sodium chloride (NaCl)	8.5
Distilled/deionized water	1 000 mL

Preparation

1. Weigh out all ingredients into a suitable container.
2. Add 1 000 mL of distilled/deionized water.
3. Dissolve and dispense into final containers.
4. Autoclave at 121 °C, 15 psi for 15 min.
5. For extraction of seeds, add 0.2 mL of sterile Tween 20 per 1 000 mL.

Storage

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

Preparation of MT (Milk Tween) agar medium

(adapted from Goszczynska and Serfontein, 1998)

Compound	g/L
A	
Proteose peptone no. 3	10.0
CaCl ₂	0.25
Tyrosine	0.5
Agar	15.0
Distilled/deionized water	500 mL
B	
Skim milk powder (Oxoid, Sigma) CCP	10.0
Distilled/deionized water	500 mL
C	
Tween 80	10.0 mL
D	
Nystatin ^a	40 mg (1 mL)
Cephalexin ^b	80 mg (1 mL)
Vancomycin ^c	10 mg (1 mL)

a, b, c Added after autoclaving

Preparation

1. Weigh out all ingredients in section A into a suitable container.
2. Add 500 mL of distilled/deionized 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. Sterilise preparations from section A, skim milk solution (section B) and Tween 80 (section C) separately at 121 °C, 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.
9. Prepare antibiotic solutions (section D).
10. Allow medium to cool to approximately 50 °C and add antibiotics.
11. Mix gently to avoid air bubbles and pour plates 22 mL per 9.0 cm plate.
12. Leave plates to dry in 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 cephalixin 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.)

Note

Cycloheximide can be used as an alternative for nystatin to control fungi. Dissolve 500 mg of Cycloheximide 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 two weeks of preparation to ensure activity of antibiotics.

Preparation of XCP1 agar medium

(adapted from McGuire *et al.*, 1986)

Compound	g/L
A	
KBr	10.0
CaCl ₂	0.25
Soluble Potato Starch	10.0
Peptone	10.0
Agar	15.0
Crystal violet (1 % aqueous)	0.15 mL
Distilled water/deionised water	1 000 mL
B	
Tween 80	10.0 mL
C	
Nystatin ^a	40 mg (1 mL)
Cephalixin ^b	10 mg (1 mL)
Fluorouracil ^c	3 mg (1 mL)
Tobramycin ^d	0.16 mg (1 mL)

^{a, b, c, d} Added after autoclaving

Preparation

1. Weigh out all ingredients in section A into a suitable container.
2. Add 1 000 mL of distilled water/deionised water.
3. Dissolve ingredients.
4. Add crystal violet.
5. Sterilise 121 °C, 15 psi for 15 min.
6. Sterilise 10 mL Tween 80 separately (section B) at 121 °C, 15 psi for 15 min.
7. Aseptically add Tween 80 to ingredients in section A.
8. Prepare antibiotic solutions.
9. Allow medium to cool to approximately 50 °C and add antibiotics.
10. Mix gently to avoid air bubbles and pour plates (22 mL per 9.0 cm plate).
11. Allow plates to dry in 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 100 mg cephalexin in 10 mL 70 % ethanol.
- ^c Dissolve 30 mg fluorouracil in 100 mL 70 % ethanol.
- ^d Dissolve 16 mg tobramycin in 100 mL 70 % ethanol.

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

Note

Cycloheximide can be used as an alternative for nystatin to control fungi. Dissolve 500 mg of Cycloheximide 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 two weeks of preparation to ensure activity of antibiotics.

Depending on the source of starch, pre-storage in the refrigerator for several days before use may result in more easily visible zones of starch hydrolysis.

Preparation of Yeast Dextrose Chalk (YDC) agar medium

(Wilson *et al.*, 1967)

Compound	g/L
Bacto Agar	15.0
Yeast Extract	10.0
CaCO ₃ (light powder)	20.0
D-Glucose (Dextrose)	20.0
Distilled/deionized water	1 000 mL

Preparation

1. Weigh out all ingredients into a suitable oversize container (i.e. 250 mL of medium in a 500 mL bottle/flask) to allow swirling of medium just before pouring.
2. Add 1 000 mL of distilled/deionized water.
3. Steam to dissolve.
4. Autoclave at 121 °C, 15 psi for 15 min.
5. Allow medium to cool to approximately 50 °C.
6. Swirl to ensure even distribution of CaCO₃ and avoid air bubbles, and 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 8-20 °C.

Prepared plates can be stored for several months provided they do not dry out.

Example of reaction mixture preparation for PCR (Audy primers)

Compound	Initial concentration	Final concentration	Volume (µl) in 20 µl
Sterile water			10.02
Promega Go Taq Buffer *	5x	1x	4
dNTP	2.5 mM each	0.2 mM	1.6
p7X4c	20 µM	0.15 µM	0.15
p7X4e	20 µM	0.15 µM	0.15
Go Taq Polymerase	5 U/µl	0.02 U/µl	0.08
DNA			4

* Concentrated PCR reaction buffer (with MgCl₂), from GoTaq DNA polymerase [Promega] (laboratories using a buffer without MgCl₂ will have to add this salt to a final concentration of 1.5 mM).

Example for visualization of PCR products**Preparation of Tris-Acetate EDTA (TAE) Buffer 1x**

Compound	mL/L
Tris-Acetate EDTA (TAE 50x)	20
Distilled/deionized water	QSP 1000 mL

Preparation of 1.5% agarose gel for electrophoresis

Compound	1 agarose gel (25x15 cm)	1 L
Tris-Acetate EDTA (TAE) 1x	300 mL	1000 mL
Agarose	4.5 g	15.0 g

Preparation

1. Make sure that the gel tray is clean and dry before use. Use the gel caster. Place the gel comb(s) in position in the gel tray.
2. Weigh out the desired amount of agarose and place in an Erlenmeyer flask with a measured amount of electrophoresis buffer, e.g. for a 300 mL gel add 4.5 g of agarose and 300 mL of 1× TAE buffer to a 500 mL flask. The larger flask ensures the agarose will not boil over.
3. Dissolve the agarose in a microwave oven. All the grains of agarose should be dissolved and the solution clear.
4. Allow the medium to cool down to approx. 60 °C and pour the gel caster.
5. After the gel is completely set carefully remove the gel comb(s).
6. Remove the gels and place them in the electrophoresis unit.
7. The same electrophoresis buffer used in the gel must also be used for the running buffer.

Note: The amount of 1.5 % agarose gel for electrophoresis to be prepared depends on the available electrophoresis apparatus of a laboratory.

References

- Audy, P., Laroche, A., Saindon, G., Huang, H. C. and Gilbertson, R.L. (1994). Detection of the bean common blight bacteria, *Xanthomonas campestris* pv. *phaseoli* and *X.c. phaseoli* var. *fuscans*, using the Polymerase Chain Reaction. *Molecular Plant Pathology*, **84**, 1185–1192.
- Birch, P. R. J., Hyman, L. J., Taylor, R., Opio, A. F., Bragard, C. and Toth, I. K. (1997). RAPD PCR-based differentiation of *Xanthomonas campestris* pv. *phaseoli* from *Xanthomonas campestris* pv. *phaseoli* var. *fuscans*. *European Journal of Plant Pathology*, **103**, 809–814.
- Boureau, T., Chhel, F., Hunault, G., Kerkoud, M., Lardeux, F., Manceau, C., Poussier, S. and Saubion, F. (2012). Procédé de dépistage de *Xanthomonas axonopodis* pv. *phaseoli*. Patent Publication 2970480, 20 July 2012.
- Darsonval, A., Darrasse, A., Durand, K., Bureau, C., Cesbron, S. and Jacques, M. A. (2009). Adhesion and fitness in the bean phyllosphere and transmission to seeds of *Xanthomonas fuscans* subsp. *fuscans*. *Molecular Plant-Microbes Interactions*, **22**, 747–757.
- Lopez, R., Asensio, C. and Gilbertson, R. L. (2006). Phenotypic and genetic diversity in strains of common blight bacteria (*Xanthomonas campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans*) in a secondary center of diversity of the common bean host suggests multiple introduction events. *Phytopathology*, **96**, 1204–1213.
- Mkandawire, A. B. C., Mabagala, R. B., Guzman, P., Gepts, P. and Gilbertson, R. L. (2004). Genetic diversity and pathogenic variation of common blight bacteria (*Xanthomonas campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans*) suggests pathogen coevolution with the common bean. *Phytopathology*, **94**, 593–603.
- Schaad, N. W., Postnikova, E., Lacy, G. H., Sechler, A., Agarkova, I., Stromberg, P. E., Stromberg, V. K., and Vidaver, A. K. (2006). Emended classification of xanthomonad pathogens on citrus. *Systematic and Applied Microbiology* **29**, 690–695.
- Simoes, T. H. N., Gonçalves, E. R., Rosato, Y. B. and Mehta, A. (2007). Differentiation of *Xanthomonas* species by PCR-RFLP of rpfB and atpD genes. *FeMS Microbiology Letters* **271**, 33–39.
- Toth, I. K., Hyman, L. J., Taylor, R. and Birch, P. R. J. (1998). PCR-based detection of *Xanthomonas campestris* pv. *phaseoli* var. *fuscans* in plant material and its differentiation from *X. c.* pv. *phaseoli*. *Journal of Applied Microbiology*, **85**, 327–336.
- Vauterin, L., Hoste, B., Kersters, K. and Swings, J. (1995). Reclassification of *Xanthomonas*. *International Journal of Systematic Bacteriology*, **45**, 472–489.
- Vauterin, L., Rademaker, J. and Swings J. (2000). Synopsis on the taxonomy of the genus *Xanthomonas*. *Phytopathology*, **90**, 677–682.