International Rules for Seed Testing 2027

Validated Seed Health Testing Methods

7-021: Detection of *Xanthomonas phaseoli* pv. *phaseoli* and *X. citri* pv. *fuscans* in *Phaseolus vulgaris* (bean) seed

Including changes and editorial corrections adopted at the Ordinary General Meeting 2026 in Calgary, Canada

Effective from 1 January 2027

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.

Published by
The International Seed Testing Association (ISTA)
Richtiarkade 18, CH-8304 Wallisellen, Switzerland

© 2027 International Seed Testing Association (ISTA)

Online ISSN 2310-3655

All rights reserved. No part of this publication may be reproduced, stored in any retrieval system or transmitted in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without prior permission in writing from ISTA.

7-021: Detection of *Xanthomonas phaseoli* pv. *phaseoli* and *X. citri* pv. *fuscans* in *Phaseolus vulgaris* (bean) seed

Host: *Phaseolus vulgaris* L.

Pathogen(s): *Xanthomonas phaseoli* pv. *phaseoli* (*Xpp*) (Smith) Constantin, Cleenwerck, Maes, Baeyen, Van Malderghem, De Vos, Cottyn (previously *X. axonopodis* pv. *phaseoli* [*Xap*] [Smith] Vauterin, Hoste, Kersters & Swings; *X. campestris* pv. *phaseoli* [Smith] Dye)
Xanthomonas citri pv. *fuscans* (*Xcf*) (Schaad *et al*.) Constantin *et al*. (previously *X. axonopodis* pv. *phaseoli* var. *fuscans* [*Xff*] Vauterin, Hoste, Kersters & Swings; *X. campestris* pv. *phaseoli* var. *fuscans* [Burkholder] Starr & Burkholder)

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

Authors: Grimault V.[1], Olivier V.[2], Rolland M.[3], Darrasse A.[4] & Jacques M. A.[4]
[1]GEVES-SNES, rue Georges Morel, B.P. 90024, 49071 Beaucouzé CEDEX, France
E-mail: valerie.grimault@geves.fr
[2]ANSES-Plant health laboratory, 7 rue Jean Dixméras, 49044 Angers CEDEX 01, France
E-mail: valerie.olivier@anses.fr
[3]BioGEVES, rue Georges Morel, B.P. 90024, 49071 Beaucouzé CEDEX, France
E-mail: mathieu.rolland@geves.fr
[4]INRA, 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

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
Version 2.2, 2012-01-01: Notice regarding review of pathogenicity test
Version 2.3, 2013-01-01: Definition of sample size
Version 3.0, 2014-01-01: New pathogenicity assay and PCR test; common name of host added
Version 3.1, 2017-01-01: Materials: numbers of Petri dishes for media deleted; Method: steps 8.1 & 8.5 modified; Reporting results revised; CCP (k) modified
Version 3.2, 2021-01-01: Safety precautions, Sample size and Methods revised
Version 3.3, 2024-01-01: Sample size, Methods and General methods revised
Version 3.4, 2025-01-01: Pathogens, Background, Materials, Methods, Critical control points, References and figure captions revised
Version 3.5, 2027-01-01: Flowchart added in Methods

Background

This method is derived from the validation studies carried out by ISTA in 2003 and 2011, in collaboration with the International Seed Health Initiative for Vegetable Crops, ISF (ISHI-Veg)(Sheppard & Remeeus, 2005). For routine testing of bean seed a combination of two complementary semi-selective media, Milk Tween™ agar (MT) and *Xanthomonas campestris* pv. *phaseoli* agar (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. phaseoli* pv. *phaseoli* (*Xpp*). In addition, both media can be used to detect both *Xpp* and *X. citri* pv. *fuscans* (*Xcf*). Although initially the morphology of *Xpp* and *Xcf* strains appear to be similar on the media, after a longer incubation the *Xcf* 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 *P. syringae* pv. *syringae*.

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 *Xpp* based on molecular methods (genetic bacterial fingerprinting in the Netherlands and pathogen specific PCRs in France). Therefore it was concluded that the pathogenicity assay used in the ISTA method, a crucial step in the *Xpp* test, is not reliable enough.

A pathogenicity assay was developed at INRA to allow a reliable characterisation of the aggressiveness of *Xpp* wild type strains and mutants (Darsonval *et al.*, 2009). A comparison study of the new pathogenicity test and primers specific for *Xpp* and *Xcf* 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*, *Xpp*, *Xcf,* 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.

Safety precautions

Dispose of all waste materials in an appropriate way (e.g. autoclaving, disinfection) and in accordance with local health, environmental and safety regulations.

Ethidium bromide

Ethidium bromide is carcinogenic. If possible, use an alternative chemical e.g. Gel Red™ (Biotium). Use ethidium bromide according to safety instructions. It is recommended to work with 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.

Ultraviolet light

Ultraviolet (UV) light must not be used without appropriate precautions. Ensure that UV protective eyewear is utilised when working with ethidium bromide.

Treated seed

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

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: known strains of both *Xanthomonas phaseoli* pv. *phaseoli* (*Xpp*) and *X. citri* pv. *fuscans* (*Xcf*) (positive controls) and of *X. vesicatoria* (negative control), or standardised reference material

Plates of milk Tween™ agar medium: 90 mm Petri dishes

Plates of XCP1 medium: 90 mm Petri dishes

Plates of yeast dextrose chalk (YDC) agar medium: for subculture (at least 1 per subsample)

Polythene bags or containers of sterile saline (0.85 % NaCl) plus Tween™ 20 (0.02 %): for soaking 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 an equivalent disinfecting product

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 (1–1000 µl)

Conventional thermocycler

Electrophoresis equipment: 1.5–2 % agarose gels

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

PCR primers (Audy *et al.*, 1994):
p7X4c: 5' GGCAACACCCGATCCCTAAACAGG 3'
p7X4e: 5' CGCCGGAAGCACGATCCTCGAAG 3'

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 achieved by swabbing/spraying equipment and gloved hands with 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.

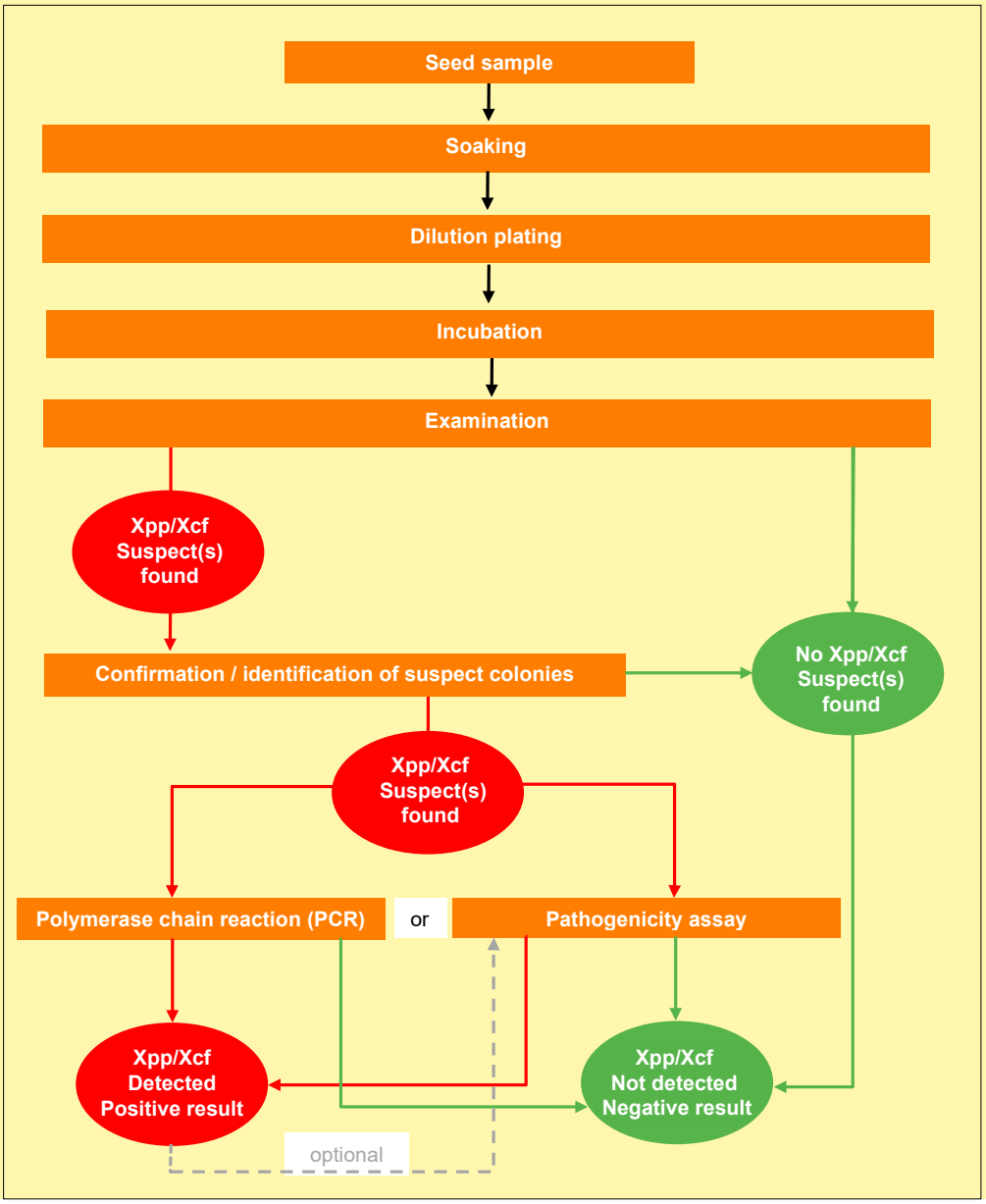
1. Count the number of seeds in a known weight. Estimate the thousand-seed weight (TSW) as:
   TSW = (weight of seeds / number of seeds) × 1000
2. Based on the estimated TSW, weigh out subsamples of the required size into new, clean polythene bags or containers.

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 ml should be equivalent to 2.5 × TSW (g); e.g. TSW = 300 g, therefore volume of saline required is 2.5 × 300 = 750 ml (Olivier & Remeeus, 2004).



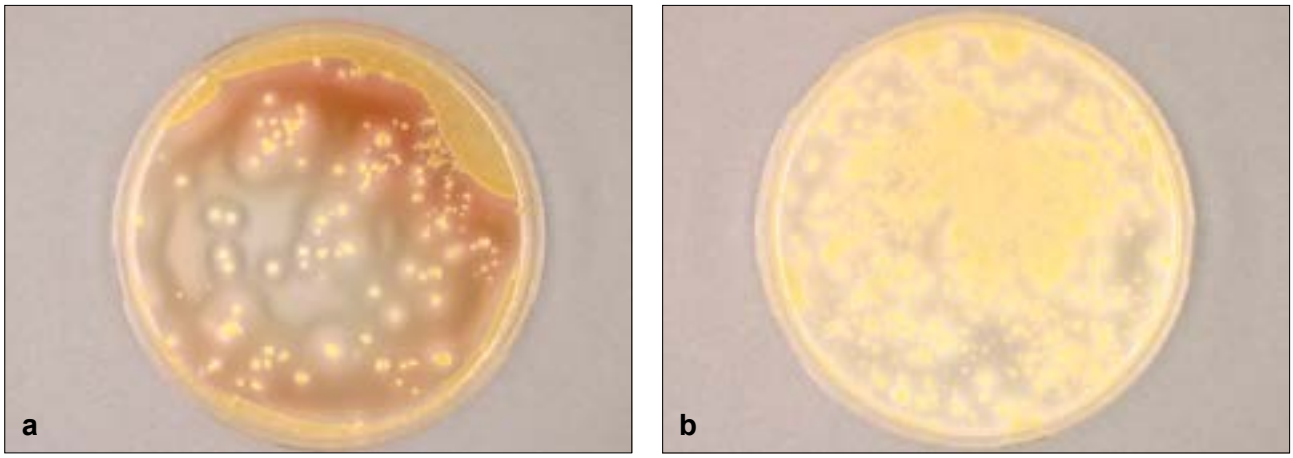


Figure 1. *Xanthomonas phaseoli* pv. *phaseoli* (*Xpp*) (colonies on MT plates after 4 d, indicated by a large clear zone of casein hydrolysis (**a**) and a smaller milky zone of Tween™ 80 lysis (**b**).

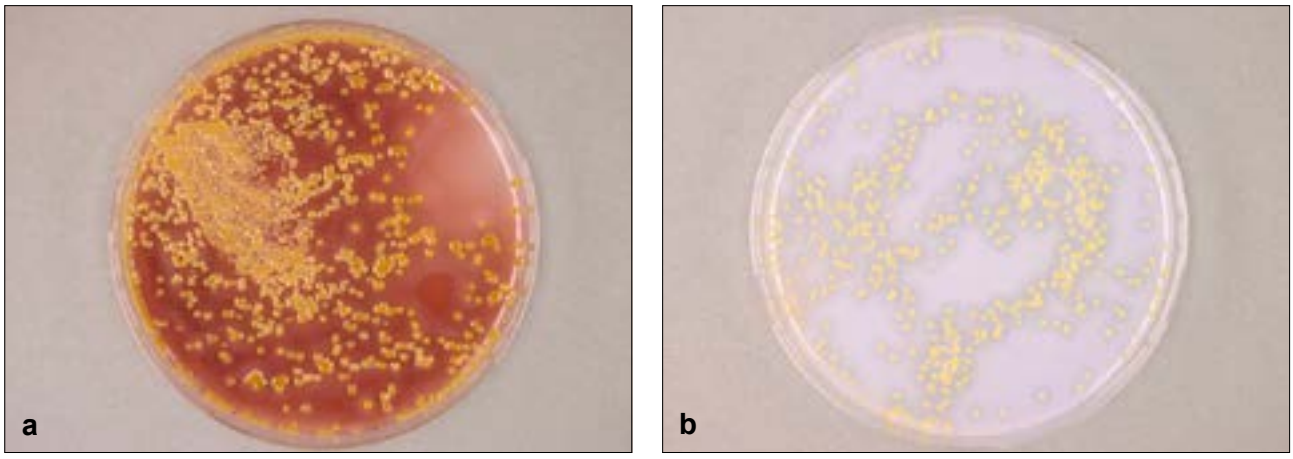


Figure 2. *Xanthomonas citri* pv. *fuscans* (*Xcf*) (**a**) and *X. phaseoli* pv. *phaseoli* (*Xpp*) (**b**) on XCP1 plates, showing a clear zone of starch hydrolysis and *X. citri* pv. *fuscans* (*Xcf*) on XCP1 showing a milky zone, after 4 d.

1.2 Soak subsamples 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 µl of each dilution and the undiluted seed extract onto plates of each of the semi-selective media (MT and XCP1) and spread over the surface with a sterile bent glass rod or equivalent (see General methods).

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

3. Positive controls (culture or reference material)

3.1 Prepare a suspension of known strains of *Xpp* and *Xcf* 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} colony-forming units (cfu) per millilitre. This may require up to seven ten-fold dilutions from a turbid suspension.

3.3 Pipette 100 µl of appropriate countable dilutions onto plates of each of the semi-selective media (MT and XCP1) and spread over the surface with a sterile bent glass rod or equivalent (see General methods).

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 semi-selective media as for samples.

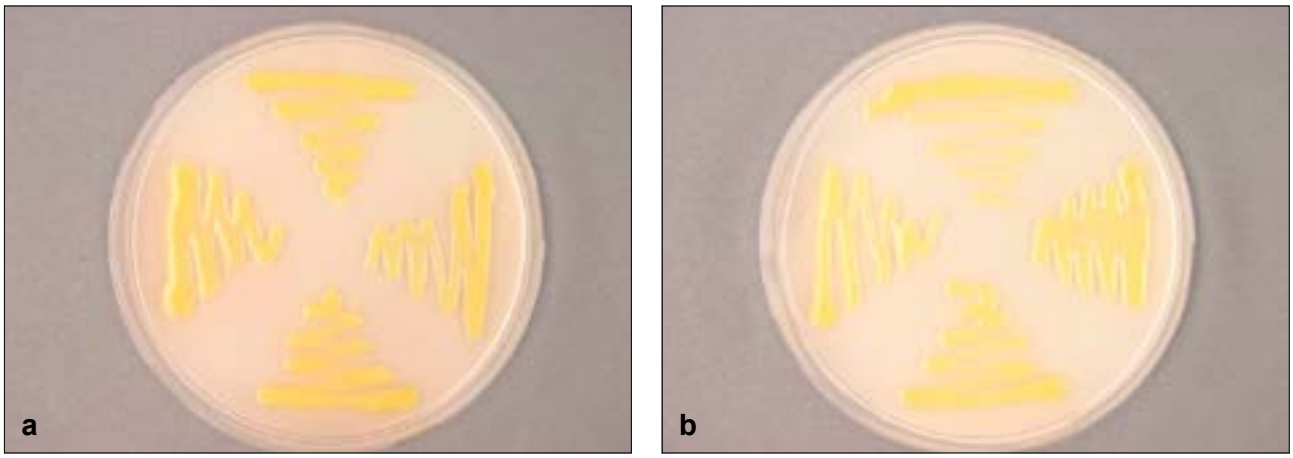


Figure 3. *Xanthomonas citri* pv. *fuscans* (*Xcf*) (**a**) and *X. phaseoli* pv. *phaseoli* (*Xpp*) (**b**), on YDC plates after 2 d are brown and yellow in appearance.

5. Examination of the plates

5.1 Examine sterility check and recovery of positive controls on both semi-selective media (MT and XCP1) (CCP).

5.2 Examine the sample plates for the presence of typical *Xpp* and/or *Xcf* colonies by comparison with the positive control plates. If necessary, estimate the number of cfu.

5.3 After 4–5 d on MT, *Xpp* 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, b). *Xcf* colonies produce a brown diffusible pigment. If not visible after 4 d, incubate for an additional day. Often the *Xcf*-type colonies show Tween™ 80 lysis.

5.4 After 4–5 d on XCP1, *Xpp* colonies are yellow, glistening and surrounded by a clear zone of starch hydrolysis (Fig. 2b). *Xcf* colonies produce a brown diffusible pigment after 5 d of incubation (Fig 2a). Often the *Xcf*-type colonies show Tween™ 80 lysis.

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/identification of suspect colonies

6.1 Subculture suspect colonies to sectored plates of YDC. To avoid the potential for 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 both *Xpp* and *Xcf* positive control isolates to a sectored plate for comparison (Fig. 3).

6.3 Incubate sectored plates for 24–48 h at 28 ±2 °C.



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

6.4 Compare appearance of growth with positive control. On YDC, *Xpp* and *Xcf* 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): option 1
(Darsonval *et al.*, 2009)

7.1 Grow seedlings of a bean cultivar known to be highly susceptible to *Xpp* and *Xcf* (e.g. 'Flavert' or 'Michelet') at 20–30 °C in small pots containing peated potting soil, until the first trifoliate leaf stage (approximately 16 days after sowing).



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

7.2 Make a 10^7 cfu/ml (CCP) suspension in distilled/deionised 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 three plants per suspect isolate.

7.5 Inoculate plants with one positive *Xpp* isolate, one positive *Xcf* isolate and two negative controls: *X. vesicatoria* and distilled/deionised 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 5–11 d (depending on when symptoms begin) after inoculation. Compare with positive and negatives controls. Typical *Xpp*/*Xcf* symptoms are water-soaked spots. Necrosis of lesions can develop and, in the case of very aggressive isolates, lead to death of tissues (Fig. 5a–d). No lesions occur on negative controls (Fig. 6)

8. Polymerase chain reaction (PCR): option 2 (Audy *et al.,* 1994)



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

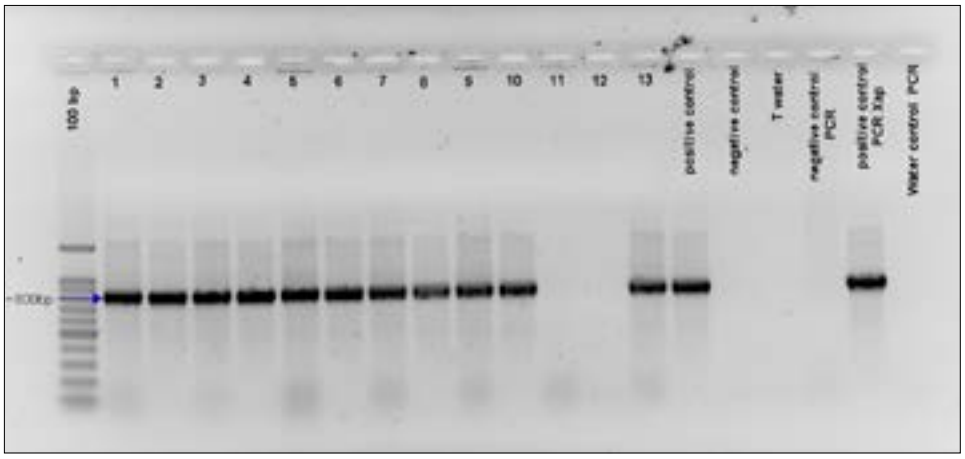


Figure 7. Agarose gel showing *Xanthomonas phaseoli* pv. *phaseoli (Xpp)*-specific band at 800 bp (Audy *et al.* (1994), primers).

8.1 Make a slightly turbid cell suspension at 10^7 cfu/ml ($OD_{600\ nm}$ approximately 0.05) in 1.0 ml sterile distilled/deionised water from the suspect colonies cultured on YDC medium, and the positive and negative controls **(**CCP). Transfer 1 ml of sterile distilled/deionised water in a separate Eppendorf tube to use as negative process control. Boil the suspension for 5 min at 95 °C for DNA extraction. Store at –20 °C until identification **(**CCP).

8.2 Use the following *Xpp*/*Xcf*-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 7-021-18, adapted by INRA)(CCP**)**. Carry out the PCR reactions in 0.2 ml thin-walled PCR tubes in a final volume of 20 µl (16 µl reaction mixture + 4 µl 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 µl of the PCR products, the negative process control and sterile water (negative PCR control) by gel electrophoresis in a 1.5 % agarose gel

in 1× Tris acetate EDTA (TAE buffer) (CCP). Include a 100 bp ladder. Stain with ethidium bromide in a bath and rinse in water.

8.6 Analyse the amplification products for a *Xpp*/*Xcf*-specific product of 800 bp (CCP) = positive identification of *Xpp*/*Xcf*; no band = negative identification (Fig. 7).

In case of a positive identification of *Xpp*/*Xcf* by PCR, a pathogenicity test can be performed as complementary information. Bear in mind that with the use of the proposed primer set, false positive results on the *Xpp*/*Xcf* lookalike bacteria *X. axonopodis* pv. *dieffenbachiae* can be obtained (Audy *et al*., 1994); however, this latter bacteria is not likely to be present on bean seed.

General methods

Preparation of ten-fold 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 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.

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.

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 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 *Xcf* 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 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).
- 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).
- 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, negative process control 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 *Xpp*/*Xcf*, under the condition that results will be validated on PCR controls.

Media and solutions

Sterile saline

(van Vuurde *et al.*, 1989)

Sodium chloride (NaCl): 8.5 g
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 1000 ml.

Storage

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

Milk Tween™ agar medium

(adapted from Goszczynska & Serfontein, 1998)

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

Section B
Skim milk powder (Oxoid, Sigma) CCP: 10.0 g
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.

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.

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 plates 22 ml per 90 mm plate.
11. Leave plates to dry in laminar flow bench or similar before use.

Storage

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

XCP1 medium

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

Section A
KBr: 10.0 g
$CaCl_2$: 0.25 g
Soluble potato starch: 10.0 g
Peptone: 10.0 g
Agar: 15.0 g
Crystal violet (1 % aqueous): 0.15 ml
Distilled/deionised water: 1000 ml

Section B
Tween™ 80: 10.0 ml

Section C
Nystatin[a]: 40 mg (1 ml)
Cephalexin[b]: 10 mg (1 ml)
Fluorouracil[c]: 3 mg (1 ml)
Tobramycin[d]: 0.16 mg (1 ml)

[a–d] Added after autoclaving. Antibiotic 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 to nystatin to control fungi. Dissolve 500 mg of cycloheximide in 10 ml 70 % ethanol, add 1 ml to cool medium.

Preparation

1. Weigh out all ingredients in section A into a suitable container.
2. Add 1000 ml of distilled/deionised water.
3. Dissolve ingredients.
4. Add crystal violet.
5. Sterilise 121 °C and 15 psi for 15 min.
6. Sterilise 10 ml Tween™ 80 separately (section B) at 121 °C and 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 90 mm plate).
11. Allow plates to dry in laminar flow bench or similar before use.

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, prestorage in the refrigerator for several days before use may result in more easily visible zones of starch hydrolysis.

Yeast dextrose chalk (YDC) agar

(Wilson *et al.*, 1967)

Agar (BD Bacto™ Agar): 15.0 g
Yeast extract: 10.0 g
$CaCO_3$ (light powder): 20.0 g
D-Glucose (dextrose): 20.0 g
Distilled/deionised water: 1000 ml

Preparation

1. Weigh out all ingredients into a suitable oversize container (e.g. 250 ml of medium in a 500 ml bottle/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. Swirl to ensure even distribution of $CaCO_3$ and avoid air bubbles, and 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 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*	5×	1×	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 $MgCl_2$), from GoTaq DNA polymerase [Promega] (laboratories using a buffer without $MgCl_2$ will have to add this salt to a final concentration of 1.5 mM).

Example for visualisation of PCR products

Tris acetate EDTA (TAE) Buffer 1×

Compound	Amount/l
Tris acetate EDTA (TAE 50×)	20 ml
Distilled/deionised water	QSP 1000 ml

1.5 % agarose gel for electrophoresis

Compound	1 agarose gel (25 × 15 cm)	Amount/l
Tris acetate EDTA (TAE 1×)	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 that 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. & 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. & 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. & Saubion, F. (2012). Procédé de dépistage de *Xanthomonas axonopodis* pv. *phaseoli*. Patent Publication 2970480, 20 July 2012.

Constantin, E.C., Cleenwerck, I., Maes, M., Baeyen, S., van Malderghem, C., de Vos, P. & Cottyn, B. (2016). Genetic characterization of strains named as *Xanthomonas axonopodis* pv. *dieffenbachiae* leads to a taxonomic revision of the *X. axonopodis* species complex. *Plant Pathology*, **65**(5), 792–806.

Darsonval, A., Darrasse, A., Durand, K., Bureau, C., Cesbron, S. & 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. & 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. & 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., & 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. & Mehta, A. (2007). Differentiation of *Xanthomonas* species by PCR-RFLP of rpfB and atpDgenes. *FeMS Microbiology Letters,* **271**, 33–39.

Toth, I. K., Hyman, L. J., Taylor, R. & 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. & Swings, J. (1995). Reclassification of *Xanthomonas*. *International Journal of Systematic Bacteriology,* **45**, 472–489.

Vauterin, L., Rademaker, J. & Swings J. (2000). Synopsis on the taxonomy of the genus *Xanthomonas*. *Phytopathology,* **90**, 677–682.

Validation references

ISTA (2012). ISTA/ISHI comparative test for method 7-021 modification for the identification of *Xanthomonas axonopodis* pv. *phaseoli* (*sensu* Vauterin *et al.,* 2000) on bean seeds. *Method Validation Reports*. International Seed Testing Association, Bassersdorf, Switzerland.