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# Detection of *Xanthomonas axonopodis* pv. *phaseoli* and pv. *phaseoli* var. *fuscans* in Bean Seed

OCTOBER 2019

**Developed by ISHI-Veg**

# Detection of *Xanthomonas axonopodis* pv. *phaseoli* and pv. *phaseoli* var. *fuscans* in Bean Seed

**Crop:** Bean (*Phaseolus vulgaris*)

**Pathogen(s):** *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans*

**Revision history:** Version 4, October 2019

## PRINCIPLE

Detection of viable *Xanthomonas axonopodis* pv. *phaseoli* (Xap) and *X. axonopodis* pv. *phaseoli* var. *fuscans* (Xapf) bacteria is typically assessed by dilution plating on two semi-selective media (MT and XCP1). Suspect bacterial colonies are then confirmed by a pathogenicity assay.

After dilution plating, a TaqMan PCR may be used for identification of the suspect bacterial colonies. The test is complete if no Xap or Xapf bacteria are detected. However, as qPCR does not prove pathogenicity, a positive TaqMan PCR must be followed with a pathogenicity assay to determine if the suspect Xap or Xapf isolate is pathogenic or not. The full method process workflow is presented in Figure 1.

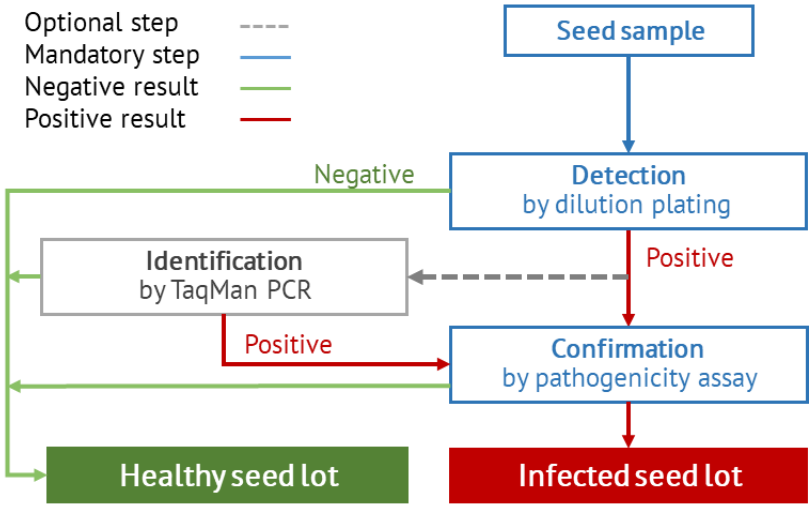


**Figure 1**: Method process workflow

## METHOD VALIDATION

The test for detecting Xap and Xapf on bean seed is an ISTA Rule (7-021) (https://www.seedtest.org/en/seed-health-methods-_content---1--1452.html). It is also an NSHS Standard A (https://seedhealth.org/seed-health-testing-methods/).

In this version a new Xap specific TaqMan assay with an internal amplification control (validated by ISHI-Veg) replaces the gel-based PCR test of ISTA Rule 7-021.

## RESTRICTIONS ON USE

This test method is suitable for untreated seed.

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

## METHOD EXECUTION

To ensure process standardization and valid results, it is strongly recommended to follow the best practices developed by ISHI-Veg for PCR and Dilution Plating Assays in Seed Health Tests. See https://www.worldseed.org/our-work/phytosanitary-matters/seed-health/ishi-veg-method-development/.

## SAMPLE AND SUB-SAMPLE SIZE

The recommended minimum sample size is 5,000 seeds with a maximum sub-sample size of 1,000 seeds.

# Protocol for detection of *Xanthomonas axonopodis* pv. *phaseoli* and pv. *phaseoli* var. *fuscans* in Bean seed

An older version of this protocol is available on ISTA's website: ISTA 7-021: Detection of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* in *Phaseolus vulgaris* (bean) seed (https://www.seedtest.org/en/seed-health-methods-_content---1--1452.html).

## SAMPLE PREPARATION

### Material

| | |
|---|---|
| Polythene bags or container | 0.85% NaCl (w/v) with 0.02% Tween™ 20 (v/v) (seed extraction buffer) |

### 1. Extraction

1.1. Suspend each subsample of seeds in sterile seed extraction buffer in a polythene bag or container. The volume of extraction buffer in mL should be equivalent to 2.5 × thousand-seed weight (TSW); e.g. if TSW = 300 g, volume of extraction buffer required is 2.5 × 300 = 750 mL.

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

## DILUTION PLATING

### Material

| | |
|---|---|
| Dilution bottles | Incubator: operating at 28–30°C |
| NaCl (0.15M) | pH meter |
| Sterile spreader | Plates of MT, XCP1 and YDC media (recipes below) |
| Pipettes and tips | Controls |

### Media recipes

#### I Milk Tween™ (MT) agar medium (adapted from Goszczynska & Serfontein 1998)

| Compound | Amount | Section |
|---|---|---|
| Proteose peptone no. 3 | 10.0 g | A |
| $CaCl_2$ | 0.25 g | A |
| Tyrosine | 0.5 g | A |
| Agar | 15.0 g | A |
| Skim milk powder (Oxoid, Sigma) | 10.0 g | B |
| Tween™ 80 | 10.0 ml | C |
| Nystatin[a] (400 mg / 10 mL 70% ethanol) | 40 mg (1 mL) | D |
| Cephalexin (800 mg / 10 mL 70% ethanol) | 80 mg (1 mL) | D |
| Vancomycin (100 mg / 10 mL 70% ethanol) | 10 mg (1 mL) | D |

[a] 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 all ingredients in section A into a suitable container.
2. Add distilled/deionized water to a final volume of 500 mL.
3. Dissolve ingredients.
4. In a separate container, dissolve skim milk powder (section B) in 500 mL distilled/deionised 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 15 psi for 15 min at 121°C.
7. After sterilization, of all components aseptically add sterilized skim milk preparation and sterilized Tween™ 80 to sterilized ingredients in section A.
8. Prepare antibiotic solutions (section D, filter sterilize when antibiotics are dissolved in water rather than 70% ethanol).
9. Allow medium to cool to approx. 50°C prior to adding antibiotics.
10. Mix gently to avoid air bubbles and pour plates 22 mL per 90 mm plate.
11. Leave plates to cool down and dry under sterile conditions.

Storage of prepared plates

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

## II XCP1 medium (adapted from McGuire et al. 1986)

| Compound | Amount | Section |
|---|---|---|
| KBr | 10.0 g | A |
| $CaCl_2$ | 0.25 g | A |
| Soluble potato starch | 10.0 g | A |
| Peptone | 10.0 g | A |
| Agar | 15.0 g | A |
| Crystal violet (1 % aqueous) | 0.15 mL | B |
| Tween™ 80 | 10.0 ml | C |
| Nystatin[a] (400 mg / 10 mL 70% ethanol) | 40 mg (1 mL) | D |
| Cephalexin (100 mg / 10 mL 70% ethanol) | 10 mg (1 mL) | D |
| Fluorouracil (30 mg / 10 mL 70% ethanol) | 3 mg (1 mL) | D |
| Tobramycin (16 mg / 100 mL 70% ethanol) | 0.16 mg (1 mL) | D |

[a] 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 distilled/deionized water to a final volume of 1000 mL.
3. Dissolve ingredients.
4. Add crystal violet (section B).
5. Sterilize at 15 psi for 15 min at 121°C.
6. Sterilize 10 mL Tween™ 80 separately (section C) at 15 psi for 15 min at 121°C.
7. Aseptically add Tween™ 80 to ingredients in section A/B.
8. Prepare antibiotic solutions (Section D, filter sterilize when antibiotics are dissolved in water rather than 70% ethanol).
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 cool down and dry under sterile conditions.

Storage of prepared plates

Store inverted plates 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 of plates in the refrigerator for several days before use may result in more easily visible zones of starch hydrolysis.

### III Yeast dextrose chalk (YDC) agar (Wilson et al. 1967)

| Compound | Amount/L |
|---|---|
| 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 |

Preparation

1. Weigh all ingredients and put them into a suitable oversized container (e.g. 250 mL of medium in a 500 mL bottle/flask) to allow swirling of the medium just before pouring.
2. Dissolve in 1,000 mL of deionized water by steaming the mix.
3. Autoclave at 15 psi for 15 min at 121°C and allow the medium to cool to approx. 50°C.
4. Swirl the bottle/flask to ensure an even distribution of CaCO3 and avoid air bubbles. Pour 22 mL on to each 90 mm plate.
5. Leave plates to cool down and dry under sterile conditions.

Storage of prepared plates

Store inverted plates in polythene bags at 8-20°C. Prepared plates can be stored for several months provided they do not dry out.

Table 1: Controls

| | |
|---|---|
| Positive Control (PC1) | A known strain of Xap |
| Positive Control (PC2) | A known strain of Xapf |
| Negative Process Control (NPC) | Extraction buffer (NaCl with Tween™ 20) |

## 1. Dilution and plating

1.1. Shake containers or polythene bags to obtain a homogenous extract before dilution.

1.2. Prepare a ten-fold dilution series from the seed extract by i.) Pipetting 0.5 mL of the extract into 4.5 mL of sterile NaCl to give a $10^1$ dilution and ii.) Pipetting 0.5 mL of the $10^1$ dilution into 4.5 mL of sterile NaCl to give a $10^2$ dilution. Vortex well all dilutions.

1.3. Pipette 100 µL of each dilution and the undiluted seed extract onto plates of the MT and XCP1 semi-selective media and spread over the surface.

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

## 2. Positive control (culture or reference material)

2.1. Prepare a suspension of a known strain of Xap, *fuscans* and non-*fuscans*, in sterile NaCl or reconstitute standardized reference material according to the supplier's instructions.

2.2. Dilute sufficiently to obtain dilutions containing approx. $10^2$ to $10^4$ CFU/mL.

2.3. Pipette 100 µL of appropriate dilutions onto plates of both semi-selective media (MT, XCP1) and spread over the surface.

2.4. Incubate plates with the sample plates.

## 3. Sterility check (Negative Process Control)

3.1. Prepare a dilution series from a sample of the extraction medium (e.g. NaCl plus Tween™ 20), without seeds. Plate each dilution on the two semi-selective media, spread over the surface and incubate (as in 1.3. and 1.4.).

## 4. Examination of the plates

4.1. Examine the sterility check and positive control plates. There should be no growth on dilution plates being used as a sterility check. The numbers of bacteria on the dilution plates should be consistent with the dilution, i.e. it should decrease approx. ten-fold with each dilution.

4.2. Examine the sample plates for the presence of typical Xap / Xapf colonies by comparison with the positive control plates. Dilution plates prepared from the positive control isolates or reference material, should give single colonies with typical morphology. The numbers of colonies on dilution plates prepared from the positive control isolates or reference material should be similar on both media.

Note: The recovery of Xapf is in general lower on MT than on XCP1.

4.3. After 4–5 days Xap colonies on MT are yellow, distinguished by two zones of hydrolysis; a large clear zone of casein hydrolysis and a smaller milky zone of Tween™ 80 lysis (Figure 2a, b). Xapf colonies produce a brown diffusible pigment. If not visible after 4 days incubate for an additional day. Often Xapf colonies show Tween™ 80 lysis.

4.4. After 4–5 days Xap colonies on XCP1 are yellow, glistening and surrounded by a clear zone of starch hydrolysis (Figure 3b). Xapf colonies produce a brown diffusible pigment after 5 days of incubation (Figure 3a). Often Xapf colonies show Tween™ 80 lysis.

4.5. The colony size and colour can differ within a sample.

4.6. Verify that the plates are readable according to the dilution plating best practices, and-record the presence of suspect colonies.

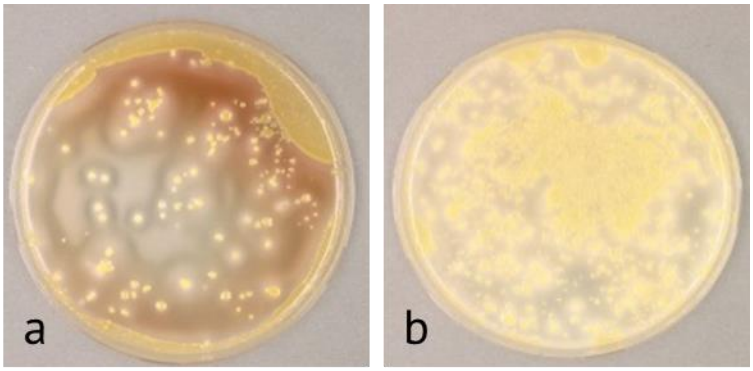


**Figure 2.** Xap colonies on MT plates after 4 days indicated by a large clear zone of casein hydrolysis (a) and a smaller milky zone of Tween™ 80 lysis (b).

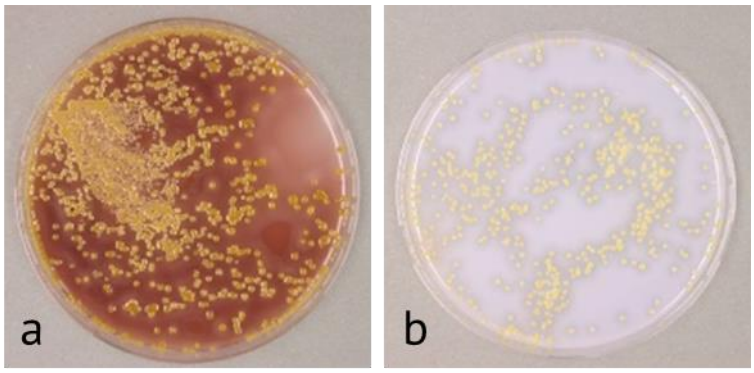


**Figure 3.** Xap colonies, fuscans (a) and non-fuscans (b), on XCP1 plates, showing a clear zone of starch hydrolysis and fuscans on XCP1 showing a milky zone, after 4 days.

## 5. Confirmation/identification of suspect colonies

5.1. 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 sub-cultured will depend on the number and variability of suspect colonies on the plate: if present, at least six colonies should be sub-cultured per subsample.

5.2. Subculture the positive control isolate to a sectored plate for comparison (Figure 4).

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

5.4. Compare the appearance of growth with the positive controls. On YDC Xap and Xapf colonies are yellow and mucoid in appearance (Figure 4). The positive control isolates or reference material should give colonies with typical morphology on YDC.

5.5. The identity of the isolates can be confirmed by the TaqMan PCR. The pathogenicity of the isolates should be confirmed by using known susceptible bean seedlings of known susceptibility by pathogenicity assay.

Note: As non-pathogenic isolates may also be present in seed lots it is essential to subculture at least the minimum number of suspect colonies specified (six per subsample), and to test the pathogenicity of all Xanthomonas-like sub-cultured isolates by a pathogenicity or PCR assay.

5.6. Record results for each colony sub-cultured.

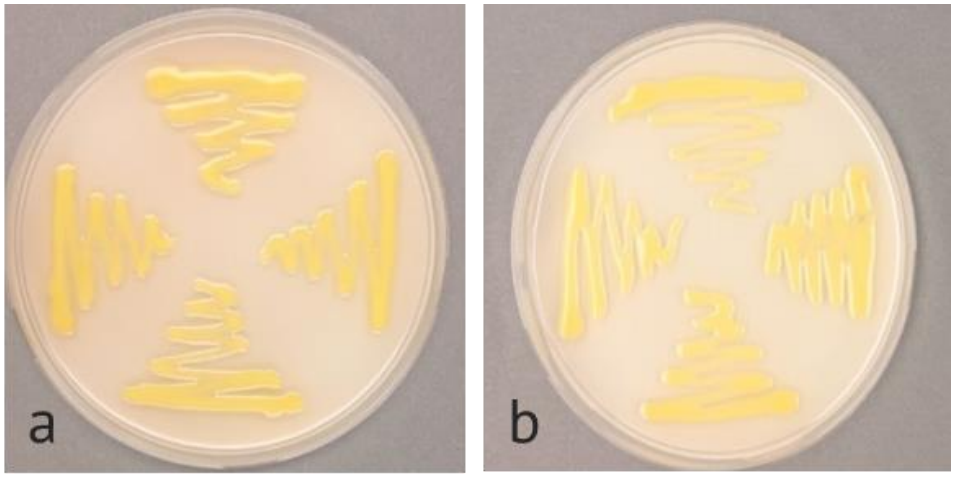


**Figure 4.** Xap colonies, fuscans (a) and non-fuscans (b), on YDC plates after 2 days are brown and yellow in appearance.

## TAQMAN PCR FOR IDENTIFICATION OF SUSPECT COLONIES

### Material

| | |
|---|---|
| DNA isolation kit e.g. Qiagen DNeasy Blood and Tissue kit | Controls |
| TaqMan PCR mix, primers and equipment | Centrifuge |
| Distilled/deionized water | Optical Density (OD) meter |

**Table 2. Controls**

| | |
|---|---|
| Positive Process Control 1 (PPC1) | Freshly prepared suspension of Xap |
| Positive Process Control 2 (PPC2) | Freshly prepared suspension of Xapf |
| Negative Process Control (NPC) | Freshly prepared suspension of non-target colony |
| Internal Amplification Control (IAC) | Universal bacterial primers (Wu et al. 2008) |
| Non Template Control (NTC) | Nucleid acid-free water |

Table 3. Primer sequences and references

| Name | Sequence | Source |
|---|---|---|
| Au-F1 | 5' – ACG GCC GGC GTC TTG TCT CT – 3' | Baldwin, 2017 |
| Au-R1 | 5' – GCC GAG GTC CGC GAG ATT CT – 3' | |
| Au-1FAM | 5' – FAM – CGT CTC TGG CTT GAC TGC GGT CGC – BHQ1 – 3' | |
| Wu-F | 5' – CAA CGC GAA GAA CCT TAC C – 3' | Wu et al., 2008 |
| Wu-R | 5' – ACG TCA TCC CCA CCT TCC – 3' | |
| Wu-Pr1 | 5' – Yakima Yellow – ACG ACA ACC ATG CAC CAC CTG – QSY - 3' | |
| Wu-Pr2 | 5' – Yakima Yellow – ACG ACA GCC ATG CAG CAC CT – QSY - 3' | |

## 1. DNA extraction

1.1. Make a slightly turbid cell suspension of at least $10^7$ CFU/mL ($OD_{600\ nm}$ approximately 0.05) in 1.0 mL sterile distilled/deionised water from the suspected cultures on YDC medium and the positive controls (Table 2). In addition, a non-target isolate should be used as a negative process control (NPC).

1.2. Heat the suspension for 5 min at 95°C for DNA extraction.

1.3. Store at –20°C until identification.

## 2. TaqMan PCR

2.1. Use the Xap specific real-time PCR primers and probes form Baldwin (2017) as well as the Internal Amplification Control (IAC) primers and probes from Wu et al. (2008) (Table 3).

2.2. During each amplification run, in addition to the PPC and NPC controls extracted in 1.1, a negative template control - molecular biology grade water - instead of DNA extract, is added.

2.3. Prepare the reaction mixture. Carry out the PCR reactions in 0.2 mL thin-walled PCR tubes in a final volume of 25 µL (20 µL reaction mixture + 5 µL boiled bacterial suspension). An example of a real-time PCR reaction composition and PCR cycling program is given in Table 4 and 5 respectively. Follow the recommended supplier's instructions for the TaqMan master mix.

2.4 Determine the cut-off values; Cq values of positive controls should consistently be lower than 30. The cut-off Cq value of the internal amplification control (IAC) should be below 35, and the expected range is to be determined by the user based on experimental data. A cut-off of Cq 35 to identify positive and negative results was used in the validation studies.

Note: In the case of universal bacterial primers, positive reactions may occur in non-template controls (NTC) due to the presence of residual DNA in Taq enzyme reagents. The IAC Cq values from reactions on suspect isolates should indicate at least a 10-fold higher concentration of bacterial DNA than the IAC Cq values from the NTC reactions; the difference between Cq values should be more than 3.3.

**Table 4: Example of a real-time PCR reaction composition**

| Component | For 1 reaction (in µL) | Final concentration |
|---|---|---|
| PCR grade $H_2O$ | 2.80 | |
| TaqMan® Universal Master Mix II (2x) | 12.50 | 1x |
| Au-F1 (10 µM) | 1.00 | 0.4 µM |
| Au-R1 (10 µM) | 1.00 | 0.4 µM |
| Au-1FAM (10 µM) | 0.20 | 0.08 µM |
| Wu-F (10 µM) | 1.00 | 0.4 µM |
| Wu-R (10 µM) | 1.00 | 0.4 µM |
| Wu-Pr1 (10 µM) | 0.25 | 0.1 µM |
| Wu-Pr2 (10 µM) | 0.25 | 0.1 µM |
| Sample | 5.00 | |
| **Total** | **25.00** | |

Note: Table 4 provides an example of the reaction mixture. However, reaction mixture, volume and conditions need to be checked and/or optimized within each laboratory on positive and negative control isolates.

**Table 5: Example of a real-time PCR cycling program**

| Step | Temperature | Duration |
|---|---|---|
| hold | 95°C | 10 min |
| 40 cycles | 95°C | 15 sec |
| | 60°C | 1 min |

## 5. Interpretation and decisions

If the specific PCR (Baldwin TaqMan) is negative and the IAC (Wu TaqMan) is positive, the suspect colony is considered not to be one of the *Xanthomonas* pathovars which cause common blight on bean. If the specific PCR is positive, the pathogenicity assay should be performed to reach a final conclusion about the identity of the suspect colony. Possible PCR outcomes and the conclusions that may be drawn are summarized in Table 6. Test results are only valid when all included controls presented in Table 2 give the expected result.

**Table 6. Interpretation and decision table for the TaqMan PCR**

| Baldwin TaqMan | Wu TaqMan | qPCR Result | Follow-up |
|---|---|---|---|
| Cq ⩽35 | | Target DNA for Xap or Xapf detected | Pathogenicity test for confirmation |
| Cq >35 or ND | Cq ⩽35 | No target DNA for Xap or Xapf detected | Negative, no follow up |
| Cq >35 or ND | Cq >35 or ND | Amplification control failure | Invalid result, repeat PCR and/or prepare a fresh isolate suspension |

## PATHOGENICITY ASSAY (Darsonval et al., 2009)

### Material

| | |
|---|---|
| Bean seedlings: susceptible to all races of the pathogen (e.g. 'Flavert' or 'Michelet') | Distilled/deionized water |

**Table 7. Controls**

| | |
|---|---|
| Positive Process Control (PC1) | A known strain of Xap |
| Positive Process Control (PC2) | A known strain of Xapf |
| Negative Control (NC) | Distilled/deionized water |
| Negative Control (NC) | *Xanthomonas vesicatoria* |

### 1. Pathogenicity assay

1.1. 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).

1.2. Make a $10^7$ CFU/mL suspension in distilled/deionized water of a culture obtained after growth (24 or 48 h), 28°C on YDC (i.e. sectored plate).

1.3. Inoculation: dip first trifoliate leaf for 30 sec in a container containing inoculum (beaker) (Figure 5).

**Figure 5.** Inoculation by dipping first trifoliate leaf for 30 sec in a beaker containing inoculum.

1.4. The number of plants which should be inoculated is 3 plants per suspect isolate.

1.5. Inoculate plants with one positive Xap isolate, and 2 negative controls: *Xanthomomas vesicatoria* and distilled/deionised water (Table 7).

1.6. Incubate at 28°C, 16 h light, 95 % RH; 25°C, 8 h dark, 95 % RH.

1.7. Record symptoms 5 to 11 days (depending on when symptoms begin) after inoculation. Compare with positive and negatives 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 (Figure 6a–d). No lesions occur on negative controls (Figure 7).

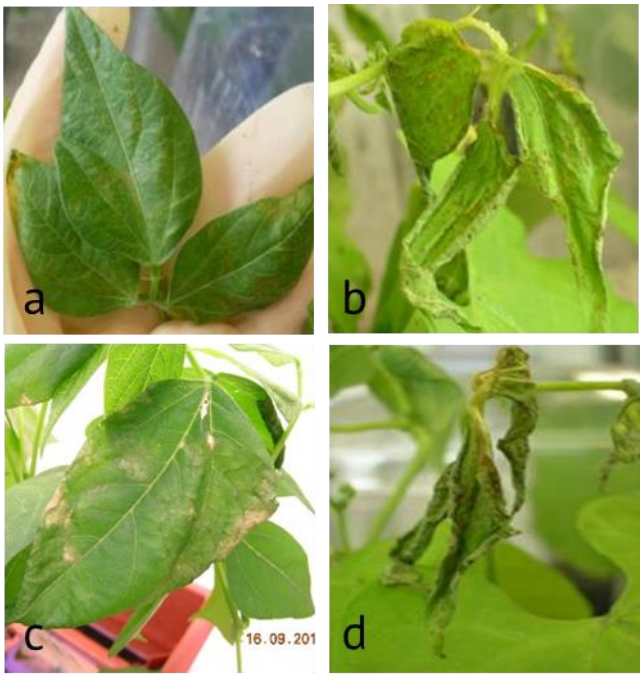


**Figure 6.** *Phaseolus vulgaris* leaves 5–11 days after inoculation with typical Xap water-soaked spots (a), necrosis (b, c) and dead tissues (d).

**Figure 7**. *Phaseolus vulgaris* leaves 5–11 days after inoculation with a negative control.

## REFERENCES

Baldwin, T. K. (2017) Development and validation of a real-time PCR assay for the identification of *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas axonopodis* pv. *phaseoli* var. *fuscans* isolates. Unpublished report available from the ISF secretariat.

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