Be 4.3 Method for testing *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* on bean and soybean seed

VERSION: 1.2	**DATE:** 4/2025
PATHOGEN: *Curtobacterium flaccumfaciens pv. flaccumfaciens* (syn: *Corynebacterium flaccumfaciens* pv. *flaccumfaciens*)	
HOST: Common bean (*Phaseolus vulgaris*), runner bean (*Phaseolus coccineus*), soybean (*Glycine max*)	
COMMON NAME: Bacterial wilt	
METHOD: Be 4.3 Selective Media Assay	
METHOD CLASS: TEMPORARY STANDARD (B)	
SAMPLE: Minimum sample size 5,000 seeds; maximum subsample of 1,000 seeds	
SUBMITTED BY: National Seed Health System	

REVISION HISTORY: Version 1.2: 4.1.2025 Updated Submitted By information Version 1.1: 3.6.2025 Sections 1, 2, and 3 added. Sample size modified, and sampling removed. Replace swab method with dilution plating and updated buffer ratio. PCR changed from optional to mandatory confirmation step, and pathogenicity test changed from mandatory to optional. Details for pathogenicity test added. Formatting updated throughout.

1. OBJECTIVE

To detect the presence or absence of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Cff) in bean seed by dilution plating on semi-selective media followed by a confirmation PCR assay.

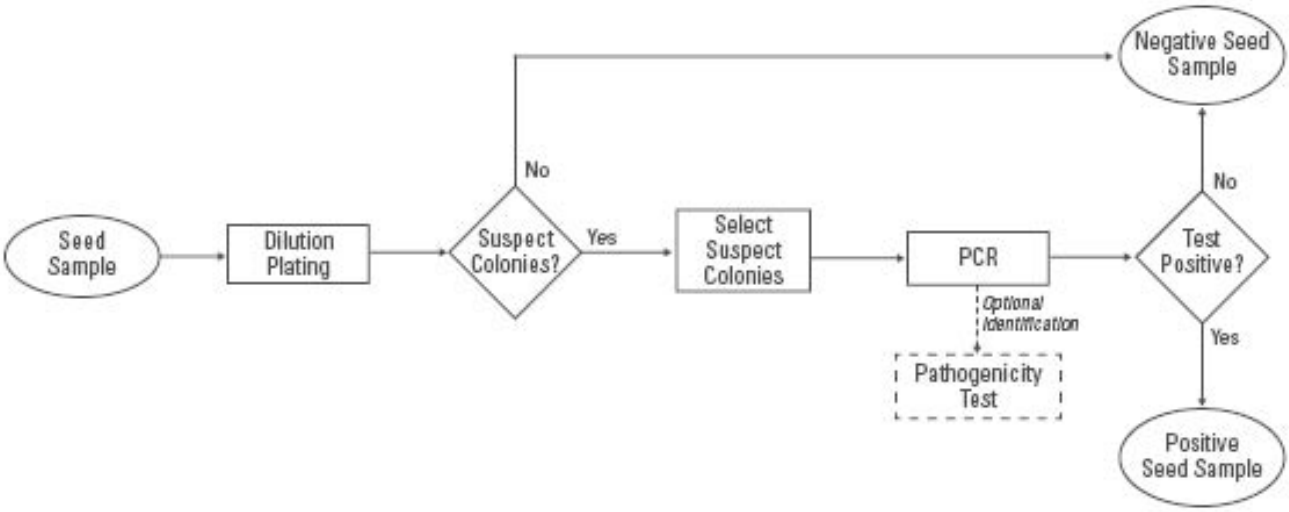
2. MATERIALS AND EQUIPMENT

Containers (1 L size)
Sterile saline with Tween™ 20
95% ethanol
Autoclave
Shaker
Vortex
Plates of CFFSM
Plates of NBY
Cff Positive control
Cold room / refrigerator at 4°C
Incubator
Plates of TTC (optional)
Biochemical tests (optional) – motility, levan, catalase, nitrate reduction, Gram stain

Susceptible bean plants (optional)
DNA extraction kit
DNA primers CffFOR2 and CffREV4 (Tegli et al., 2002)
Taq DNA polymerase
dNTPS (all 4), Tris-HCL, KCl, $MgCl_2$
PCR controls
PCR thermocycler
Centrifuge
Agarose gel electrophoresis
Gel imaging system

3. METHOD PROCESS WORKFLOW

Method Be 4.3 Process Workflow



4. METHOD

4.1. Sample Preparation and Extraction

4.1.1. Add seed subsamples to containers

A. Suspend each subsample of seeds in Sterile saline plus Tween™ 20 buffer. The volume of seed extraction buffer in mL should be adjusted according to the number of seeds used to keep an equivalent ratio (TSW x 2.5 mL).

B. Use sterile equipment and containers.

4.2. Dilution Plating

4.2.1. Soak subsamples overnight (16-18 hours) at 5°C.

4.2.2. Agitate containers or bags to obtain a homogenous extract before dilution.

4.2.3. Prepare a ten-fold dilution series from the seed extract. For example, 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. Vortex to mix (10^{-2} dilution).

4.2.4. Pipette 100 µL of each dilution and the undiluted seed extract onto plates of each of the semi-selective media, CFFSM and NBY agar (See “Recipes”) and spread over the surface with a sterile bent glass rod.

4.2.5. Streak positive Cf pv. flaccumfaciens controls onto both NBY and CFFSM media, and a negative

control of the sterile PBS buffer.

4.3. Incubation

4.3.1. Plates are incubated at 24-28°C for 5 to 6 days.

4.4. Bacterial Identification

4.4.1. Colony morphology:

A. On NBY: Slow growing, small (1-4 mm) flat colonies appear after 2-3 days. Colonies are raised, with round smooth edges. Colony pigmentation varies from yellow-cream to yellow. Orange, pink, and blue (rare) variants have also been described.

B. On CFFSM: Circular with yellow to orange coloration, casein hydrolysis and a slight fading of the dye around the colonies.

4.4.2. If no presumptive positive colonies are found, the seed lot is considered negative.

4.4.3. Presumptive positive colonies are streaked onto Nutrient Agar (NA) or NBY for further analysis. Proceed to 4.5 PCR assay and the optional biochemical / and pathogenicity tests.

4.5. PCR assay. Tegli et al., 2002

4.5.1. Prepare bacteria DNA using an appropriate method.

4.5.2. Primers CffFOR2 (5'-GTT ATG ACT GAA CTT CAC TCC-3') and CffREV4 (5'-GAT GTT CCC GGT GTT CAG-3') (Tegli et al., 2002) are used to amplify bacterial DNA. Follow best practices for PCR testing. PCR master mixes or slight modifications to the thermocycler conditions used in your laboratory must be proven to show equivalency.

A. PCR reaction control samples must be used and produce expected outcomes. An internal control is needed, such as universal bacteria primers (Roberts and Koenraadt, 2019), 1052F 5'-GCA TGG TTG TCG TCA GCT CGT-3' and BacR 5'-TAC GGC TAC CTT GTT ACG ACT T-3' resulting in a 441 bp amplicon. Failure of control(s) voids the PCR assay and the test must be re-run.

B. Guidance for controls and PCR best practices can be found at ISHI's website in the document "Best Practices for PCR Assays in Seed Health Tests".

Reagent	**Final Conc.**
Primer CffFOR2	0.5 µM
Primer CffREV4	0.5 µM
Tris-HCl	20 mM
KCl	50 mM
$MgCl_2$	1.5 mM
dNTP - dATP	100 µM
dNTP - dCTP	100 µM
dNTP - dGTP	100 µM
dNTP - dTTP	100 µM
Taq DNA polymerase	1 Unit
DNA extract	10 ng
Total	25 µl

4.5.3. PCR Amplification Conditions:

	Temperature	Time
Denaturation	94 °C	3 minutes
PCR cycling (30 cycles)	94 °C	1 minute
	62 °C	45 seconds
	72 °C	30 seconds
Final Extension	72 °C	5 minutes

4.5.4. Run gel electrophoresis. The seed lot will be considered positive if a band at 306 bp is observed in any replicate sample and the control reactions are as expected.

4.5.5. The seed lot will be considered negative if no band is observed at 306 bp.

4.6. Pathogenicity Testing (optional, adapted from Venneman, J., et. al., 2024)

4.6.1. Use a susceptible bean cultivar (e.g., Blue Lake 274, Blue Lake 47, Contender, Proton, Ferrari, or Borlotto). The plants should not have more than two trifoliate leaves at inoculation, but should have at least one trifoliate leaf.

4.6.2. Suspend a 2-day old Cff colony in 1 mL of 10 mM PB or PBS to create a 10^8 CFU/ml suspension.

4.6.3. Inoculate at least 3 individual plants per Cff isolate by either method:

A. Pipette a 10 µl drop of the bacterial suspension on the node of the first leaves and introduce the inoculum in the stem by pricking with an entomological pin through the droplet into the vascular strand of the stem.

B. Using a syringe with needle, inject the bacterial suspension into 2 places through the stem of the plant, 3 cm above the ground and beneath the first two leaves. Try to leave a small drop of suspension on both sides of the stem.

4.6.4. Intake of the droplets is facilitated when bean plants are slightly dehydrated.

4.6.5. Run the test with positive and negative control plants inoculated as described above. Use a Cff strain with validated pathogenicity for the positive control plants. Use 10 mM PB or PBS for the negative control plants.

4.6.6. Incubate plants at 22 – 28°C with at least 14 hours of light/day. In general, avoid dry air and temperatures above 32°C for several days which will damage the bean leaves and mask symptoms.

4.6.7. Keep the plant substrate moist but not wet. Do not supply water overhead to avoid splash dispersal of the isolates.

4.6.8. Examine at 7 to 14 days for flaccidity in leaves and wilt. If no wilting is observed, pathogenicity was not confirmed.

4.6.9. If wilting is observed, re-isolate the Cff from the main vein of a symptomatic leaf on NBY.

4.6.10. Identify the re-isolate through further molecular or morphological evaluations.

4.7. Suspected isolation plates are moved to differential media. (optional)

4.7.1. On TTC: Colony color is red for Cff.

4.8. Additional Biochemical Tests. (optional)

4.8.1. Motility: motile

4.8.2. Levan: negative

4.8.3. Catalase: positive

4.8.4. Nitrate: not reduced

4.8.5. Gram stain: positive

5. RECIPES

5.1. NBY (nutrient broth yeast medium modified with cycloheximide) – Schaad et al., 2001.

Ingredient	Quantity
Deionized water	1,000 mL
Nutrient broth	8 g
Yeast extract	2 g
Glucose	5.0 g
K_2HPO_4	2 g
KH_2PO_4	0.5 g
Agar	15 g

Mix ingredients, autoclave and cool to 50°C to 55°C. Add the following ingredients and mix:

Ingredient	Quantity
1 M $MgSO_4$ solution – autoclaved separately	1 mL
Cycloheximide (in 70% ethanol)	100 mg

5.2. TTC agar medium. Schaad et al., 2001.

Ingredient	Quantity
Deionized water	1,000 mL
Glucose	10 g
Peptone	10 g
Casamino acid (casein hydrolysate)	1 g
Agar	18 g

Mix ingredients, autoclave and cool to 50°C to 55°C. Add the following ingredient and mix:

Ingredient	Quantity
1% w/v 2,3,5 triphenyl tetrazolium chloride solution – autoclaved separately	1 mL

5.3. Nutrient agar medium – from Difco (Becton, Dickinson and Company)

Ingredient	Quantity
Deionized water	1,000 mL
Difco nutrient agar	23 g

5.4. Sterile saline plus Tween™ 20

Ingredient	Quantity
Deionized water	1000 mL
NaCl	8.5 g
Tween 20	0.2 mL

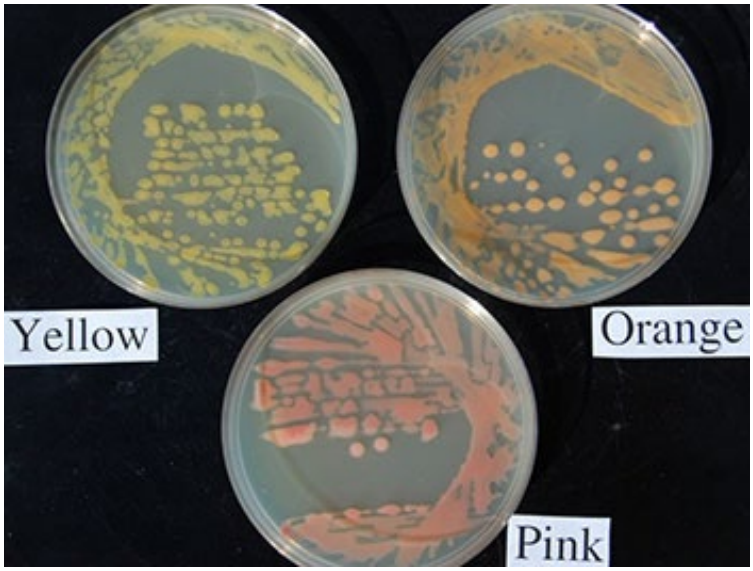
5.5. CFFSM agar medium (Maringoni et al., 2005)

Ingredient	Quantity
Distilled water	1,000 mL
Peptone	5 g
Meat Extract	3 g
Sucrose	5 g
Agar	15 g

Mix ingredients, autoclave and cool to 50°C to 55°C. Add the following ingredients and mix:

Ingredient	Quantity
Skim milk powder (5 g in 50 ml dH2O, autoclave separately)	5 g
Congo red (use 2 ml stock solution)	0.05 g
Chlorothalonil (use 1 ml stock solution)	0.01 g
Thiophanate methyl	0.01 g
Nalidixic acid (use 1 ml stock solution)	0.01 g
Nitrofurantoin (use dry powder)	0.01 g
Oxacillin	0.001 g
Sodium azide – optional (use dry powder)	0.001 g

6. PICTURES



Colony morphology on NBY agar medium. Photo courtesy Dr. Robert Harveson, Professor/Extension Plant Pathologist, University of Nebraska.

Figure 1. Cultural characteristics of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* isolated from bean seeds on the CFFSM culture medium.

Colony morphology on CFFSM agar medium. Maringoni et al., 2005.

Primary bean leaves infected by CFF. (Università di Bologna; photo from EPPO).

7. REFERENCES

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