



VERSION: 1.0	DATE: 12/2012
PATHOGEN: <i>Curtobacterium flaccumfaciens</i> pv. <i>flaccumfaciens</i> (syn: <i>Corynebacterium flaccumfaciens</i> pv <i>flaccumfaciens</i>)	
HOST: common bean (<i>Phaseolus vulgaris</i>)	
COMMON NAME: bacterial wilt	
METHOD: Be 4.3 Selective Media Assay (University of Idaho)	
METHOD CLASS: TEMPORARY STANDARD (B)	
SAMPLE: See Below	

PROCEDURE:

I. Seed Sampling

Samples must be taken by an officially USDA accredited entity, or state authority. Sample size requirements are below. Please label samples with lot number and variety.

Sample size required:

5 lb for each 10,000 lb in the lot or, for lot sizes:

201 to 1,000 – 3.0 lb sample

50 to 200 lb – 2.0 lb sample

26 to 50 lb – 1.5 lb sample

15 to 25 lb – 1.0 lb sample

10 to 14 lb – 0.5 lb sample

less than 10 lb – take appropriate representative sample

II. Sample Preparation

A. Divide seeds into 3 subsamples of 2000 seeds each. Add seeds to 1L beakers.

B. Wash seeds under running tap water until water runs clear, and rinse 1 time with sterile water. Use mesh screens to cover seeds.

C. Add cleaned seeds to sterile 2L Erlenmeyer flasks and add 1500ml Phosphate Buffered Saline (PBS) buffer (See "Recipes").

D. Re-sterilize any equipment (mesh screens, funnels, etc.) with 95% ethanol between lots of seed. Autoclave flasks to be used each time.

III. Sample Extraction

A. Flasks are incubated at 24-28 C° and agitated occasionally. All beans should be submerged in the buffer.

B. Take first sample after 6-8 hours of incubation.

1. Flask is agitated, and a sterile cotton swab is swirled through the flask and streaked out directly onto CFFSM and NBY agar (See "Recipes"). Streak 2 plates per flask.

2. Alternatively, agitate flask and plate 10ul of each subsample on CFFSM and NBY agar (See "Recipes"). Spread the 10ul evenly across the plate with a sterile glass rod.

C. Take second sample at 8 to 16 hours.

1. Flask is agitated, and a sterile cotton swab is swirled through the flask and streaked out directly onto CFFSM and NBY agar (See "Recipes"). Streak 2 plates per flask.

2. Alternatively, agitate flask and plate 10ul of each subsample on CFFSM and NBY agar. Spread the 10ul evenly across the plate with a sterile glass rod.

D. Take third sample at 24 to 32 hours.

1. Flask is agitated, and a sterile cotton swab is swirled through the flask and streaked out directly onto CFFSM and NBY agar. Streak 2 plates per flask.

2. Alternatively, agitate flask and plate 10ul of each subsample on CFFSM and NBY agar. Spread the 10ul evenly across the plate with a sterile glass rod.

E. Streak positive *Cf pv. flaccumfaciens* controls onto both NBY and CFFSM media, and a negative control of the sterile PBS buffer.

F. After isolation, beans are destroyed, and flasks are cleaned and autoclaved.

IV. cubation

Plates are incubated at 24-28 C° for 4 to 5 days.

V. Bacterial Identification

A. Colony morphology:

1. 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 and pink variants have also been described.
2. On CFFSM: Circular with yellow to orange coloration, casein hydrolysis and a slight fading of the dye around the colonies.

B. Presumptive positive colonies are streaked onto Nutrient Agar (NA) or NBY for further analysis and biochemical/pathogenicity testing. Then proceed to one or more of the following biochemical / serological / molecular and pathogenicity tests:

1. Suspected isolation plates are moved to differential media. (optional)
 - a. on TTC: Colony color is red for CFF.
2. Serology-agglutination. (optional)
 - a. Place 5ml of 0.9% saline solution into sterile glass vial. (Saline solution: 0.9g NaCl, 100ml sdH₂O, 2-3 drops formalin)
 - b. Dip sterile swab into saline solution and rub over plate of suspected isolate (grown 24-48 hr on NA), and also run a separate positive CFF control.
 - c. Swirl inoculated swabs in saline solution.
 - d. Pipette 2 drops antiserum for CFF into each of 5 wells.
 - e. Add 2 drops of antigen-saline solution into each well.
 - f. Incubate on rotary shaker for 20 minutes.
 - g. Examine under dissecting scope for agglutination, positive isolates will show fluffy precipitate.
 - h. Confirm positive by diluting 5ml saline with antigen to 9ml and retest.

3. PCR. Tegli et al., 2002 (optional)

a. Bacterial DNA is extracted using a Puragene DNA Isolation Kit (Gentra System Inc. Minneapolis, MN) and the Instagene Matrix (Bio-Rad, Hercules, CA) according to manufacturer's instructions.

b. Primers CffFOR2 and CffREV4 (Tegli et al., 2002) are used to amplify bacterial DNA. Total reaction volume is 25ul, with 10ng DNA, 20 mM Tris-HCl, 50 mM KCl, 1.5mM MgCl₂ 100uM of each dNTP, 0.5 uM of each primer pair, and 1 U of Taq DNA polymerase.

c. Amplification conditions are as follows: initial denaturation at 94 C° for 3 minutes, 30 cycles of denaturation (1 minute at 94 C°), primer annealing (45 seconds at 62 C°), and primer extension (30 seconds at 72 C°). A final extension is done at 72 C° for 5 minutes.

d. 2.5ul aliquots of PCR product are resolved by electrophoresis in 1.4% agarose gels and show a 306 bp fragment.

4. Additional Biochemical Tests. (optional)

a. Motility: motile.

b. Levan: negative.

c. Catalase: positive.

d. N2: not reduced.

e. Gram stain: positive.

5. Pathogenicity testing.

a. Suspend 2 day old colony to 10⁵–10⁶ cfu/ml bacteria, and inoculate stem of susceptible plant (such as great northern cultivar US1140, navy bean cultivar AC Morden 003, navy bean cultivar AC Cruiser, plus others) at axillary bud by inserting dissecting needle approximately 4mm deep and placing 5-10ul suspension on the wound. Inoculate 2-4 plants.

b. Run with positive and negative control plants.

c. Examine at 7-11 days for wilt.

d. Re-isolate suspected pathogen on NBY.

RECIPES:

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

1L water

8g nutrient broth

2g yeast extract

2.5g glucose

2g K₂HPO₄

0.5g KH₂PO₄

15g agar

Autoclave and cool to 50°C to 55°C, then add:

1 ml 1M MgSO₄ solution – autoclaved separately.

100 mg cycloheximide (in 70% Ethanol)

2. TTC media. Schaad et al., 2001.

1L water

10g glucose

10g peptone

1g casamino acid (casein hydrolysate)

18g agar

Autoclave and cool to 50°C to 55°C, then add:

1ml 1% w/v 2,3,5 triphenyl tetrazolium chloride solution, autoclaved separately.

3. Nutrient Agar – from Difco (Becton, Dickinson and Company)

23g Difco Nutrient Agar

1L water

4. Phosphate Buffered Saline (PBS) (pH = 7.4)

8g NaCl

0.4g KH₂PO₄

1.15g Na₂HPO₄

0.4g KCl

1L H₂O

5. CFFSM (Maringoni et al., 2005)

5g Peptone

3g Meat extract

5g Sucrose

15g Agar

1L Distilled water

Autoclave and cool to 50°C to 55°C, then add;

Skim milk powder* 5g (5gm in 50ml dH₂O, autoclave separately)

Congo red* 0.05g (use 2ml stock solution)

Chlorothalonil* 0.01g (use 1ml stock solution)

Thiophanate methyl* 0.01g

Nalidixic acid* 0.01g (use 1ml stock solution)

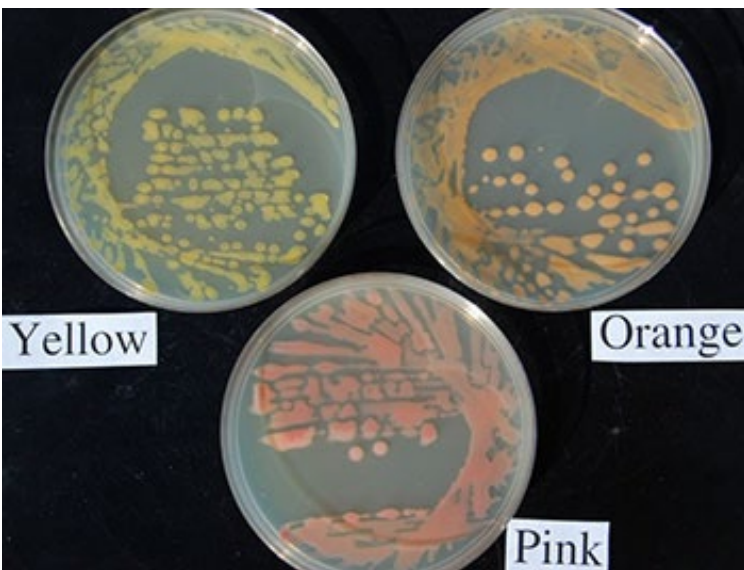
Nitrofurantoin* 0.01g (use dry powder)

Oxacillin* 0.001g

Sodium azide* 0.001g (use dry powder)

*added after autoclaving the basal medium.

PICTURES:



Colony morphology on NBY. 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.
Maringoni et al., 2005



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

REFERENCES:

CAB International. 2004. Crop Protection Compendium. Wallingford, UK: CAB International.

Carlson, R. R. and Vidaver, A. K. 1982. Taxonomy of *Corynebacterium* plant pathogens, including a new pathogen of wheat, based on polyacrylamide gel electrophoresis of cellular proteins. *International Journal of Systematic Bacteriology*. 32(3):315-326.

EPPO. 1994. *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* field inspection and seed-testing methods. *Bulletin OEPP (EPPO)*. 24: 329-331.

Hayward, A. C. and Waterston, J. M. 1965. *Corynebacterium flaccumfaciens*. CMI Description of pathogenic fungi and bacteria, No. 43. Wallingford, UK: CAB International.

Hsieh, T. F., Huang, H. C., Mündel, H. H., Conner, R. L., Erickson, R. S. and Balasubramanian, P. M. 2005. Resistance of Common Bean (*Phaseolus vulgaris*) to Bacterial Wilt Caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*. *Journal of Phytopathology*. 153(4):245-249.

Maringoni, A. C., Camara, R. C., and Souza, V. L. 2006. Semi-selective culture medium for *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* isolation from bean seeds. *Seed Sci. Technol.* 34: 117-124.

Maringoni, A. C. and Camara, R. 2006. *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* detection in bean seeds using a semi-selective culture medium. *Braz. J. Microbiol.* [online]. 37(4):451-455.

Schaad, N., Jones, J. and Chun, W. 2001. Laboratory guide for identification of plant pathogenic bacteria. Third Edition. APS Press, St Paul MN.

Tegli, S., Sereni, A. and Surico, G. 2002. PCR-based assay for the detection of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in bean seeds. *Lett. Appl. Microbiol.* 35: 331-337.