VERSION: 1.0	**DATE:** 12/2012
PATHOGEN: Acidovorax avenae ssp. citrulli (syn: A. citrulli)	
HOST: Cucurbits	
COMMON NAME: bacterial fruit blotch (BFB)	
METHOD: Cb 1.5 CSP Labs Seedling PCR	
METHOD CLASS: TEMPORARY STANDARD (B)	
SAMPLE: 10,000 seeds; sub samples: large seeds = 2,000 seeds (squash, gourd, pumpkin), small seeds = 2,500 seeds (watermelon, melon, cucumber)	

PROCEDURE:

1. Treatment of seeds:

a. Confirm seed lot and sample weight required for testing 10,000 seed. Weigh out 100 seeds to be used to calculate germination and place in sealed sample bag.

b. Place remaining 9,900 seed in large sample bag.

c. Thiram 42S is diluted to 1:10 for safer handling and is used at 25 ul per gram seed for slurry treatment. This method delivers 2500 ppm of thiram (formulation basis). Add thiram slurry as directed by label to large sample bag containing 9,900 seed. Shake sample bag to evenly distribute seed coating. Ensure proper personal protective equipment is worn.

2. Preparation of Domes:

a. New 1020 flats (no hole) fitted with raised plastic lids (domes) are stored in clean, dry areas and are assembled to create high relative humidity during germination.

b. 8 or 10 trays and domes are required for testing 10,000 small or large seeds, respectively.

3. Planting of seeds:

a. Add 1000 CC vermiculite to each flat, 8 flats for small seed, 10 flats for large seed.

b. The first 1020 tray contains a thick plastic barrier 4×4 (small seed) or 5×4 (large seed) which separates 100 seed from the remaining sample and is used to calculate germination (see Figure 1).

c. Broadcast 1,250 (small) or 1,000 (large) seed per flat.

d. Confirm that no seeds are within the germination barrier, if seeds are found remove them. Open the small sample bag containing 100 non-treated seed and disburse seed within the germination barrier.

e. Cover seeds with an additional 1000 CC of vermiculite.

f. Use tap water for each flat (850 ml for melon/cucumber, 1250 ml for watermelon and 1380 ml for squash/gourd/pumpkin).

g. Place domes on top of flat and secure with binder clips. Additional binder clips may be necessary for larger seed due to rapid growth that exerts a lifting force on dome.

h. Gloves are changed throughout processing after handling treated seed and adding vermiculite/water, as well as after cleaning bench spaces with 70% EtOH. Glove change at every step within a sample seems unnecessary from a cross contamination point of view.

i. To avoid cross contamination, gloves are changed between samples. Chemical resistant gloves are worn when handling treated seed.

4. Incubation:

a. Samples are incubated in a growth room for 12 days. Environment is kept at 80°F (±3°F) with a 12 hr photoperiod. Temperature variation is minimized by use of circulating fans.

b. Each flat is placed side by side, not stacked, on grow room bench under direct light (Figure 2). Light source is centered above dome approximately 7 inches and contains 2 bulbs (60 watt bulbs (equivalent to 23-30 watt CFL or 16-20 watt LED lights).

c. Flats are not disturbed during incubation period.

5. Examination of seedlings:

a. After incubation, transfer flats from growth room to processing room.

b. Remove clips and domes and examine health of seedlings noting interference from damping off, melting, and fungi and symptoms of BFB (Figure 3).

c. Determine germination of 100 seeds in designated flat. At least 20% germination is required to validate PCR results.

6. Isolation from symptomatic tissue and use of immune-strip: (optional)

a. Seedlings with symptoms of BFB can be tested for the presence of Ac with Agdia immunostrip kit and/or removed for tissue isolation if further investigation is needed.

b. Confirm identity of suspects via biochemical assays and PCR.

7. Extraction of pathogen:

a. The contents of two flats are combined into a large plastic extraction container. Each extraction container is designated as a single sub-sample. Therefore 4 or 5 extraction containers are required for small and large seed, respectively.

b. Coarsely chop seedlings so that each hypocotyl gets cuts twice (Figure 4).

c. Add tap water (enough to soak all seedlings, Figure 4).

d. Incubate for 1 hour to extract bacteria from the seedlings.

e. Draw 80 ml of seedling extract through a filter to ensure vermiculite and plant debris are removed. Transfer extract from each extraction container into pre-labeled 85 ml centrifuge tube.

f. Centrifuge at 12,000 rpm for 6 minutes.

g. Decant most of the extract, leaving 2-3 mls, and re-suspend pellet by vortexing.

h. Submit 500 ul of seedling extract for PCR for each sub-sample. Retain 500 ul of seedling extract as a backup and store at -20°C

8. DNA extraction: (See appendix 1 for detailed procedure)

a. Centrifuge 500 ul extract.

b. Isolate DNA using standard procedure.

c. Elute DNA into 100 ul RNase free water.

9. Check for PCR inhibitors:

a. Non target DNA is used for Internal control (IC) check. The non-target DNA is isolated from *Acidovorax cattleye* (Acat) and its concentration adjusted to obtain a Ct value of 28±3. To check for PCR inhibitors, 10 ul of Acat cells (IC) is added into each 500 ul extract before DNA extraction. After qPCR, if Ct values are out of expected range, DNA is re-extracted and qPCR is rerun. See appendix for detailed procedure.

10. PCR for Acidovorax avenae subsp citrulli:

5 ul DNA is tested by qPCR with duplex reaction using Primer 54 & 847 and Ct value determined. (See appendix 2 for detailed procedure).

11. Clean up:

All plant material is disposed of in a sanitary landfill. All lab equipment used to extract the pathogen is washed and sanitized by soaking in 1% sodium hypochlorite (10,000 ppm available chlorine).

12. Reporting:

Results are reported as positive or negative. The sample yielding >35 Ct value is considered Negative. For positive samples, additional information is provided to include number of sub samples positive and Ct values.

APPENDIX 1: Bacterial DNA extraction protocol (1.5 ml format)

Sample collection step

1. Turn on dry bath to 65°C.

2. Collect 500 µl of bacterial suspension in 1.5 ml tube.

3. Spike 10 µl of Acat IC cells into each tube.

4. Centrifuge at 13,100 x rpm for 3 min.

5. Carefully draw 400 µl of supernatant out without disturbing pellet.

6. Add 1-3 Zirconium oxide HD 3 mm ball.

7. Vortex in FastPrep at 1,000 RPM for 3 min.

8. Centrifuge at 4,000 rpm for 30 sec. Use 1.5 ml adapter.

Lysate step

9. Add 250 µl of GHL buffer. Add following chemicals just before use to 10 ml of GHL buffer: Proteinase K (10 mg/ml) – 20 µl RNase (100 mg/ml) – 20 µl Lysozyme (50 mg/ml) – 100 µl

10. Vortex in FastPrep 96 at 500 RPM for 2 min.

11. Incubate at 65°C for 1 HR in dry bath. Precipitation step

12. Add 250 µl of GHP Buffer.

13. Vortex in FastPrep 96 at 500 RPM for 2 min.

14. Incubate at -20 °C for 20 min.

Cleaning step

15. Load 500 µl to Homogenizer. Use cut tip if necessary.

16. Centrifuge at 13,100 x rpm for 5 min. Binding step

17. Transfer 400 µl to new 2.0 ml tube without disturbing the pellet.

18. Add 600 µl of GHB buffer. Mix well by inverting the tubes upside down.

19. Load 400 µl of supernatant onto a DNA binding column. Use 1.5 ml vacuum adapter.

20. Vacuum for 1-2 min (approx. -25 inHg).

21. Repeat step 20 & 21. Wash step

22. Add 500 µl of S3 buffer.

23. Vacuum for 1-2 min (approx. -25 inHg).

24. Add 500 µl of 75% Ethanol.

25. Vacuum for 1-2 min (approx. -25 inHg).

26. Repeat 75% Wash step a total of 3 times.

27. Transfer DNA binding column to cap less tubes.

28. Centrifuge at 13,100 x rpm for 3 min to remove extra ethanol.

29. Transfer from DNA binding column to a new collection tube.

Elution step

30. Load 50 µl of RNase free water pre-warmed at 65°C on to the DNA binding column.

31. Centrifuge at 13,100 x rpm for 2 min. Keep the flow through.

32. Repeat Elution step 31-32.

33. Transfer 100 µl of DNA to 96 well DNA collection plate.

34. Store DNA at -20 °C.

BUFFERS USED:

GHL buffer	
DI Water	400 ml
1M Tris HCl pH 7.4 (100 mM)	100 ml
5M NaCl (500 mM)	100 ml
0.5M EDTA (50 mM)	100 ml
20% SDS (1.0%)	50 ml
1M Sodium sulfite (52 mM)	52 ml
1N NaOH (200 mM)	2 ml
Bring volume to 1,000 ml.	

GHP buffer	
DI Water	400 ml
Acetic Acid (2.4 M)	141.2 ml
Potassium acetate (3.6 M)	294.42 g
Bring volume to 1,000 ml.	

GHB buffer	
DI Water	150 ml
Guanidine hydrochloride (6 M)	286.9 g
Bring pH down to 4.5 with Acetic acid.	
Bring volume to 500 ml with DI Water.	

S3 Buffer	
Guanidine hydrochloride (2M)	95.33 g
95% Ethanol	350 ml
Warm 10-15 sec in microwave to dissolve.	
Bring the volume to 500 ml with 95% Ethanol.	

APPENDIX 2: PCR Protocol

Sample consists of 1 ml of bacterial concentrate from seedling extract. 500 ul is used for DNA isolation. The remainder 500 ul is saved as a backup sample. Purified DNA is eluted in 100 ul RNase free water and checked for presence of PCR inhibitors. Spike checked for the presence of PCR inhibitors. Non target DNA is used for Internal control (IC) check. The non-target DNA is

isolated from *Acidovorax cattleye* (Acat) and its concentration adjusted to obtain a Ct value of 28 ± 3. To check for PCR inhibitors, 10 ul of Acat IC is added into each 500 ul extract before DNA extraction. After qPCR, if Ct values are out of expected range, DNA is re-extracted and qPCR is rerun.

Primers
Internal control (IC) – inhibitor check
847-F (Acat 2-F) – TGT AGC GAT CCT TCA CAA G
847-R (Acat 2-R) – TGT CGA TAG ATG CTC ACA AT
847-P (Acat 1-Pr) – HEX – CTT GCT CTG CTT CTC TAT CAC G – Zen-IABLK

Target (Acidovorax citrulli) detection (primers patented, US patent 6,146,834)
54-F (Aac-f3) – cct cca cca acc aat acg ct
54-R (Aac-r2) – tcg tca tta ctg aat ttc aac a
54-P (Aap2) – 6FAM – cgg tag ggc gaa gaa acc aac acc – Zen-IABLK

PCR conditions (Bio-Rad CFX 384)
95C/2 min,
45 Cycles, 95C/3 sec, 60C/30 sec

Reaction set up:

Component	qPCR (1 reaction)	1X
PCR Water	2.6 ul	
GO Taq Probe qPCR Master Mix 2x (Promega)	10.0 ul	1X
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PCR Water	2.6 ul	
GO Taq Probe qPCR Master Mix 2x (Promega)	10.0 ul	1X
847-F	0.4 ul	150nM
847-R	0.4 ul	150nM
847-P	0.4 ul	100nM
54-F	0.4 ul	300nM
54-R	0.4 ul	300nM
54-P	0.4 ul	200nM
Sample DNA5	5.0 ul	
Total	20.0 ul	

FIGURES:

Figure 1. Thick 4×4 germination barrier in the first 1020 flat. Small seeds (100) will be placed within the barrier and germination counted after 12 day incubation period.

Figure 2. 1020 flats with secured domes are placed in growth room under direct light for 12 days.

Figure 3. Symptoms of water soaked lesions on cotyledons observed during extraction process. Left to right: watermelon, gourd, squash and melon.

Figure 4. Left: Seedlings are chopped for extraction. Right: Chopped Seedlings are suspended in water.