Diagnostics of *Dickey dianthicola*

These protocols are for detection and identification of *Dickeya* sp., particularly *D. dianthicola*, in plant samples. Please note that these methods have not been validated by PPQ. They are the results of a literature survey and experience.

Isolation from tissue

Sampling from potato tuber

1. Gently wash tubers with 10% bleach to remove excess soil. Rinse with water and damp dry.
2. Using a disposable razor blade, cut into the stolon tip of the tuber and look for infected tissue. Take sample tissue from the margins of the infected area (see image below). Including both necrotic and healthy tissue in the sample, cut enough material for approximately 1.0 g of tissue. Chop into pieces on sterile weigh boat.
3. Place ~100 mg of the sample into a Q-Biogene Lysing matrix tube to be used for nucleic acid extraction using the Qiagen DNeasy Plant Mini Kit.
4. Place ~500 mg into a mesh extraction bag (e.g., Agdia or Bioreba) with 3 mL of 50 mM phosphate buffer (PB, pH 7.2, containing 4.26 g Na2HPO4 and 2.72 g KH2PO4/liter) and thoroughly crush sample.
    a. Pipette out 500 µL of liquid and place into 1.5 mL microcentrifuge tube to be used for bacterial isolation.

Sampling from potato stem

1. Wipe external part of stem with 10% bleach wipe to remove excess soil. Rinse with water and damp dry.
2. Using a disposable razor blade, remove a ~5 cm section of stem containing both diseased and healthy tissue, near the crown region if possible.
3. Chop the tissue using a new disposable razor blade on a sterile weight boat.
4. Place ~100 mg of tissue into a Q-Biogene Lysing matrix to be used for nucleic acid extraction using the Qiagen DNeasy Plant Mini Kit.
5. Place ~500 mg of the tissue into a mesh extraction bag with 3 ml 50 mM PO_4 buffer and crush sample.
    a. Pipette out 500 µL of liquid and place into 1.5 mL microcentrifuge tube to be used for bacterial isolation.

DNA Extraction

Thoroughly disrupt tissue in a lysing matix tube using a FastPrep machine, bead- beater, or similar. Extract DNA according to Qiagen instructions.

Figure 1: Locations circled for infected tuber and stem sampling.

Bacterial Isolation

1. Allow crushed material to soak in buffer for 5-10 minutes at room temperature.
2. Using a sterile inoculating loop, take one loop of suspension and streak onto three petri plates:
    a. Tryptic Soy Agar (TSA)
    b. Nutrient Agar (NA)
    c. Crystal violet pectate medium (CVP) [Sonia N. Humphris et al., *Diagnosis of Pecobacterium and Dickeya spp.* 2015]
3. Optional: Pipette 20-40 µL of the sample suspension onto three fresh petri plates (TSA, NA, and CVP) and streak the liquid fully across the media surface using a sterile plastic spreader. Store sample suspension at 4˚C.
4. Seal each plate with parafilm. Incubate at 32˚-37˚C for 24 hours. Store sample suspension at 4˚C.
5. Examine plates for single colonies, ideally about 2 mm or larger in diameter.
6. Select colony using a 1 µL sterile plastic loop and place into 1.5 mL microcentrifuge tube with 200 µL 10% DMSO 100 mM Tris.
7. Incubate samples at 95˚C for 2 min. Immediately place on ice.
8. Store at 4˚C for short term, and -20˚C for long term storage.

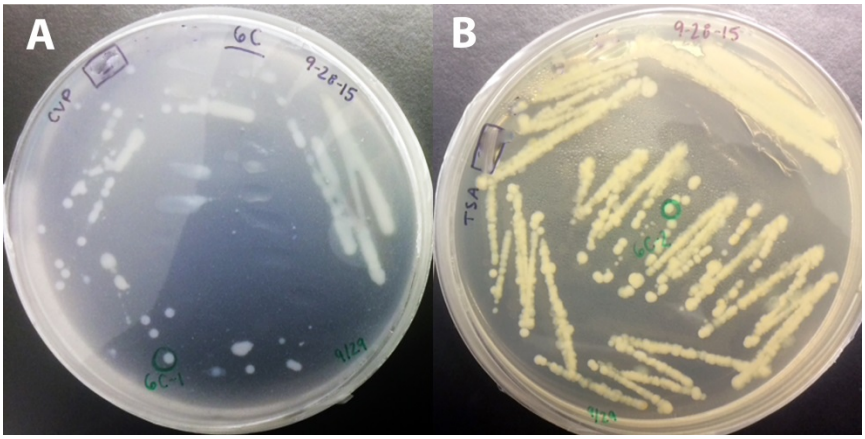

Figure 2: Dickeya colonies on select media for bacterial isolation. **A)** CVP **B)** TSA

Conventional PCR Assays

Master Mixes for PCR

1. Keep all reagents and samples on ice when working.
2. Prepare Master Mix following Table 1 and aliquot 23 µL into each 0.2 mL PCR tube.
3. Add 2.0 µL of undiluted sample DNA for a total reaction volume of 25 µL. Each PCR run should include two controls: *Dickeya sp.* control and non-template control (NTC-MG water)

Table 1: For all PCR reactions use the following master mix:

Reagent	Amount for 25 µL reaction volume
Nuclease-Free H_20	9.5 µL
GoTaq® Green Master Mix (Promega M712)	12.5 µL
6 µM Primer mix (Forward+Reverse)	1.0 µL

Diagnostics

NOTE: although contemporary publications describing bacterial characterization using MLSA (multi-locus sequence analysis) describe using DNA from pure bacterial colonies, we have had good success targeting the genetic locations in the following table (fliC, dnaX, and gyrB) using total DNA extracted using a Qiagen kit which has tested positive for presence of Dickeya using one or both of the PCR screening tests (DF/DR, pelADE1/pelADE2). When successful, this method vastly reduces the time and labor required to determine a sample positive for Dickeya. Occasionally, more than one bacterial species will be amplified, which results in an unreadable sequence when direct sequencing is used. For this reason, we recommend testing with all three MSLA primer sets in order to obtain at least 2 sequences with the required homology to *D. dianthicola*.

1. To screen suspect samples for *Dickeya* sp., run primer sets pelADE1/2 and/or DF/DR conventional PCR on all samples.
2. Using samples that gave positive results for either or both pelADE1/2 / DF/DR tests, continue testing using conventional PCR primer sets flic1/2, dnaXF/R, and gyrBF/R.
3. For each run, use 10 µl of PCR product on 1.5% agarose gel using 1X TAE buffer. Run at 100V for 60 min. and stain with ethidium bromide for visualization.

Table 2: Conventional PCR primers

Primer Name	Sequence 5'-3'	Amplicon Size (bp)
pelADE1	GAT CAG AAA GCC CGC AGC CAG AT	420
pelADE2	CTG TGG CCG ATC AGG ATG GTT TTG TCG TGC	
DF	AGA GTC AAA AGC GTC TTG	133
DR	TTT CAC CCA CCG TCA GTC	
fliC1	TAT CAA CAG CGC CAA AGA CAA CGC	650
fliC2	ACG GCT CAT GTT GGA TAC TTC GTT	
dnaXF	TAT CAG GTY CTT GCC CGT AAG TGG	535
dnaXR	TCG ACA TCC ARC GCY TTG AGA TG	
gyrBF	TAA GTT YGA CGA YAA CTC STA YAA RGT	974
gyrBR	CCC CTT CCA CCA GGT ASA GTT C	

Table 3: Conventional PCR cycling conditions

Primers	Step 1	Step 2	Step 3
pelADE1/2	94˚C for 2 min	34 cycles: 94˚C for 30s, 72 ˚C for 90s	72 ˚C for 5 min
DF/DR	94˚C for 2 min	30 cycles: 94˚C for 20s, 62˚C for 90s, 72 ˚C for 90s	72 ˚C for 5 min
fliC1/2	95˚C for 1 min	35 cycles: 95˚C for 20s, 55˚C for 30s, 72 ˚C for 45s	72 ˚C for 5 min
dnaXF/R	94˚C for 3 min	35 cycles: 94˚C for 60s, 59˚C for 60s, 72 ˚C for 120s	72 ˚C for 5 min
gyrBF/R	94˚C for 4 min	35 cycles: 94˚C for 60s, 56˚C for 60s, 72 ˚C for 120s	72 ˚C for 10 min

Evaluation of Samples

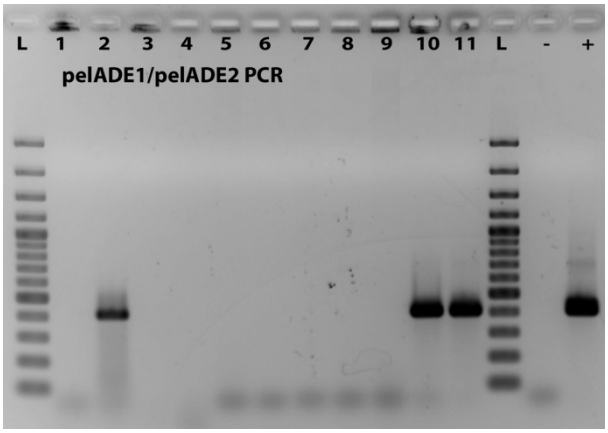

Figure 3: pelADE1/pelADE2: Conventional PCR assay of Dickeya suspect samples. Lanes 2, 10 and 11 are positive.

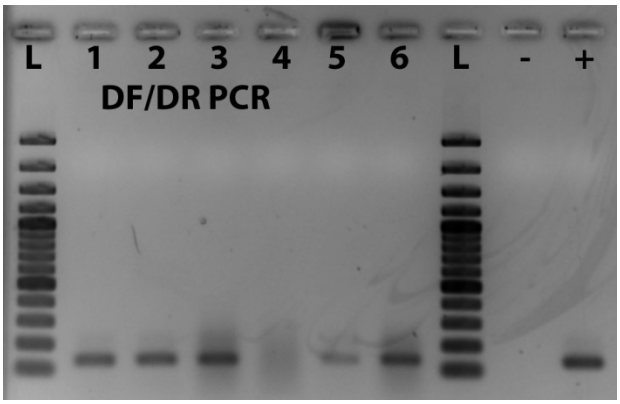

Figure 4: DF/DR: Conventional PCR assay of suspect Dickeya samples. Lanes 1, 2, 3, 5, and 6 are positive.

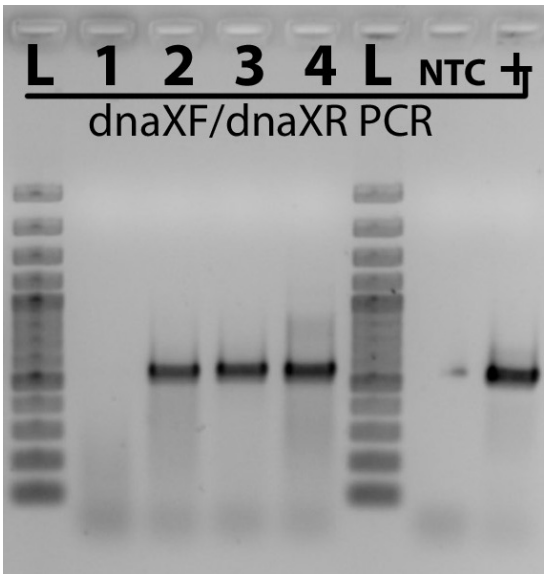

Figure 5: dnaXF/dnaXR: Conventional PCR assay of suspect Dickeya samples. Lanes 2, 3, and 4 are positive.

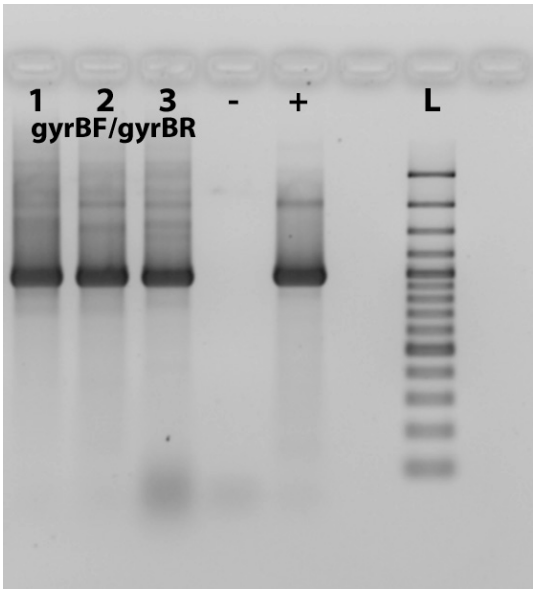

Figure 6: gyrBF/gyrBR: Conventional PCR assay of suspect Dickeya samples. Lanes 1, 2, and 3 are positive.

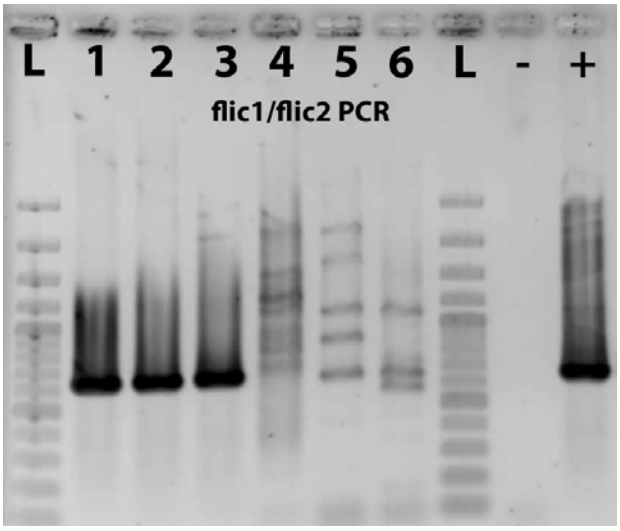

Figure 7: flic1/flic2: Conventional PCR assay of suspect Dickeya samples. Lanes 1, 2, and 3 are positive.

Sequence analysis of directly sequenced PCR products

1. PCR products from fliC, dnaX, and gyrB that show a positive result matching the positive control with no contamination in the NTC can be directly sent for sequencing. Please note that presence of a PCR product of the apparent correct size is not proof that Dickeya DNA has been amplified.
2. In order to determine positive for Dickeya, BLAST aligned consensus sequences against the following accession # and % match.
    a. *fliC:* ≥ 98% homology with accession KF442436.1: positive for *D. dianthicola*
    b. *dnaX:* ≥ 98% homology with accession LC043164.1: positive for *D. dianthicola*
    c. *gyrB:* ≥ 98% homology with accession AOOK01000040.1: positive for *D. dianthicola*

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