VERSION: 2.0	**DATE:** 09/2023
PATHOGEN: Pantoea stewartii (syn: Erwinia stewartii)	
HOST: Maize (Zea mays)	
COMMON NAME: Stewart's wilt	
METHOD: Mz 10.1 ELISA (Enzyme-linked immunosorbent assay) and confirmation SYBR PCR assay	
METHOD CLASS: STANDARD (A)	
SAMPLE: 400 seeds	

REVISION HISTORY:
10/2020 – measurement correction on Step #10.
09/2023 – addition of SYBR and Conventional PCR procedures.

MATERIALS:

ELISA

- Antiserum: suitable for detection of Pantoea stewartii (e.g. Agdia, AC Diagnostics, DSMZ, etc.)
- Distilled or deionized water
- Extraction and ELISA buffers
- Microtiter plates: 96 well plates, suitable for ELISA
- Grinder: capable of grinding seeds to fine flour
- Microplate spectrophotometer capable of operation at 405 nm

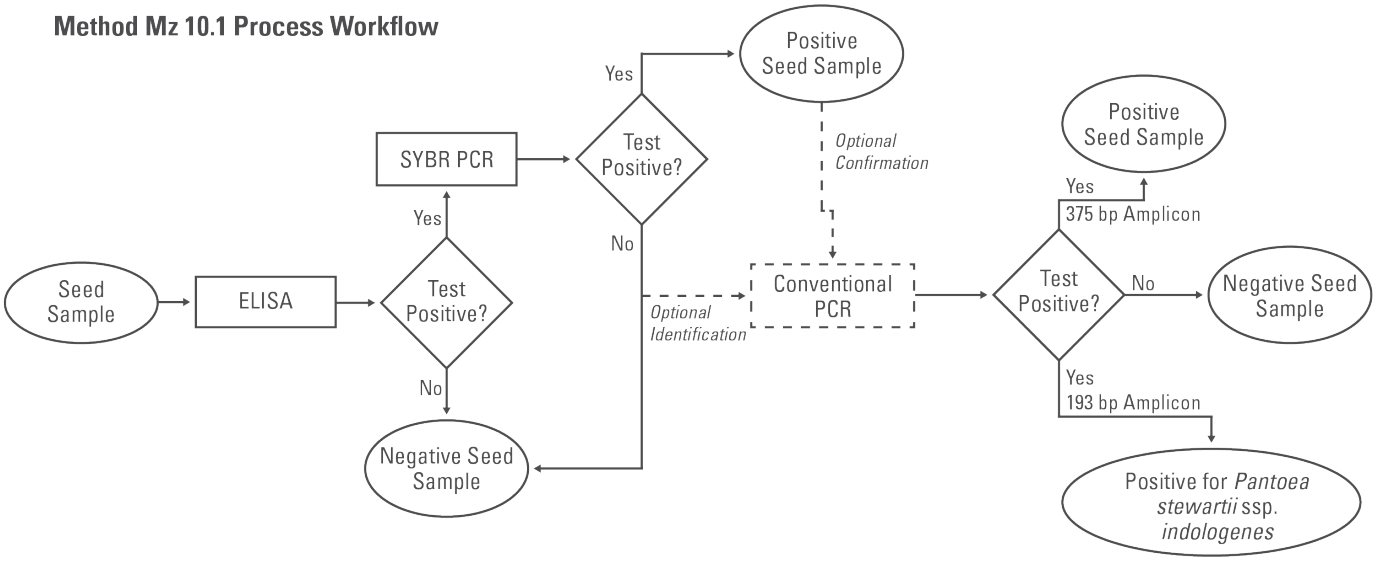
SYBR PCR

- Centrifuge
- TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0)
- DNA Extraction - CTAB OR Dneasy Blood & Tissue Kit (Qiagen)
- Primers - Cps AB 2313F and Cps R
- PowerUp SYBR Green Master Mix 2X
- PCR-grade water
- *Pss* positive control, negative control, and non-template control
- qPCR thermocycler
- Spectrophotometer (nanodrop) - optional

Conventional PCR (optional)

- Primers - Cps F and Cps R
- Go Taq Green Master Mix 2X (Promega)
- Gel Electrophoresis equipment
- Agarose gel
- SyBR-Safe or GelRed stain
- Gel Imaging System

Method Mz 10.1 Process Workflow



PROCEDURE:

Preparation of ELISA plate:

1. Dilute Pantoea stewartii coating antibody in coating buffer as defined by the supplier.
2. Add 100 µl of coating solution to each well.
3. Cover plates to minimize evaporation.
4. Incubate plates for 4 hours in a humid box or overnight at 4°C.

Extraction of the bacterium:

1. Grind 4 replicates of 100 corn seeds, per sample and add 100 ml of General Extraction Buffer.
2. OR, place 4 replicates of 100 corn seeds into 100ml of General Extraction Buffer; soak overnight at room temperature, then grind.
3. Disinfect the grinder between samples by rinsing with distilled water, followed by a rinse with a laboratory or hospital standard detergent, then a final rinse with distilled water.

Running the ELISA kit:

1. Empty coated plate and wash 3 times with PBS-Tween.

2. Pipette 100 µl of seed extract into appropriate wells.

3. Add positive and negative controls to the plate.

4. Incubate plates for 2 hours in a humid box at room temperature, overnight at 4°C or as defined by the antibody supplier.

5. Prepare enzyme conjugate dilution according to supplier directions, use within 10 minutes of preparation.

6. Remove seed extracts from the plate and wash the plate 3 to 5 times with PBS-T.

7. Add 100 µl of diluted conjugate solution to each well.

8. Incubate for 2 hours at room temperature.

9. Wash plate 6 to 8 times with PBS-T.

10. Prepare PNP substrate solution (10 mg para-nitrophenylphosphate in 10 ml substrate buffer (makes enough for 96 wells, adjust volumes if necessary).

11. Add 100 µl substrate solution to each well.

12. Incubate in the dark for 30 to 60 minutes or as specified by the manufacturer.

Evaluating ELISA plates:

Evaluate results using a spectrophotometer plate reader at 405 nm. The threshold for a positive reaction on the plate reader should be greater than 2X the negative control.

PROTOCOL-SYBR PCR Detection of Pantoea stewartii subsp. stewartii in maize seeds

This PCR assay is used as a follow-up to ELISA testing- NSHS method Mz 10.1 (Lamka et al. 1991) to confirm ELISA-positive or suspect-positive (i.e. elevated absorbance) results. If ELISA results were negative, testing is complete and PCR follow-up is not needed.

PROCEDURE:

1-Preparation of samples of untreated corn seeds:

1.1 DNA extraction follows a suspect-positive ELISA test where the ground seed was already mixed with ELISA extraction buffer. The ELISA extract can be refrigerated overnight or frozen for later use.

1.2 Remove 25 ml from each subsample and place into a 50 ml centrifuge tube.

1.3 Centrifuge tubes for 5 minutes at 1000 g (slow spin) to pellet corn debris. Carefully pour supernatant into a clean 50 ml tube.

1.4 Centrifuge the supernatant for 10 minutes at 5000-6000 x g (fast spin) to pellet bacteria.

Note: Be careful to avoid breaking/cracking centrifuge tubes.

1.5 Completely remove the supernatant with a pipette and discard liquid.

1.6 Add 567 µl of TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer to the pellet and mix by repeated pipetting. Carefully transfer the contents to a new 1.5 ml tube.

1.7 Proceed with DNA extraction and purification. Two options are available: CTAB method - Section 2 or Qiagen DNeasy Blood & Tissue Kit Extraction –Section 3.

2-(DNA purification option 1) – CTAB

Note: *It is likely that the protocol will not be completed in one day. The method is quite sensitive, so be careful to avoid cross-contamination. An example protocol follows:*

- **Day 1**: Sample preparation (steps 1.1 to 1.7 above). Refrigerate samples overnight.
- **Day 2**: DNA extraction. Refrigerate the samples overnight.
- **Day 3**: qPCR- SYBR.

Solutions:

- TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0)
- 10% sodium dodecyl sulfate (SDS) – (recipe in *Annex A*)
- 20 mg/ml proteinase K
- 5 M NaCl (recipe in *Annex A*)
- CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) (recipe in *Annex A*)
- 24:1 chloroform/isoamyl alcohol
- 25:24:1 phenol/chloroform/isoamyl alcohol
- 70% Isopropanol (2-propanol) – store in -20F freezer.
- 70% ethanol

2.1 Add to the 567 µl-pellet-TE suspension (from step 1.7): 30 µl of 10% SDS and 3 µl of 20 mg/ml proteinase K. Mix thoroughly and incubate 1 hour at 37℃.

2.2 Add 100 µl of 5 M NaCl and mix thoroughly.

2.3 Add 80 µl of CTAB/NaCl solution. Mix thoroughly and incubate 10 min at 65℃.

2.4 Add an approximately equal volume (0.7 to 0.8 ml) of chloroform/isoamyl alcohol, mix thoroughly, and spin 10 min in a microcentrifuge at 10,000 rpm.

2.5 Remove aqueous, viscous supernatant to a fresh microcentrifuge tube, leaving the interface behind. Add an equal volume of phenol/chloroform/isoamyl alcohol to the newly transferred liquid, mix thoroughly, and spin in a microcentrifuge for 10 min at 10,000 rpm.

2.6 Transfer the supernatant to a fresh tube. Add 0.6 ml of cold isopropanol to precipitate the

nucleic acids. Shake the tube back and forth until a stringy white DNA precipitate becomes clearly visible. At this point, the precipitate can be pelleted by centrifuging for 10 min at 10,000 rpm at room temperature. Completely remove and discard the supernatant with a pipette.Note: A DNA precipitate is not always visible.

2.7 Wash the DNA pellet with 70% ethanol to remove residual CTAB and re-spin 5 min at 10,000 rpm to pellet it. Carefully remove the supernatant and briefly dry the pellet in a lyophilizer OR after decanting the last bit of ethanol, let air dry the tubes inverted upside down.

2.8 Re-dissolve the pellet in 50 µl sterile water. Refrigerate.

3- (DNA purification option 2): DNeasy Blood & Tissue Kit (Qiagen).

Preparation:

- Buffer ATL and Buffer AL may form precipitates during storage. If necessary, warm to 56°C for 5 min until the precipitates have fully dissolved.
- Buffer AW1 and Buffer AW2 are supplied as concentrates. For a new bottle, add the appropriate amount of ethanol (96–100%) as indicated on the bottle to obtain a working solution.
- Mix Buffer AW1 before use by inverting several times.

PROCEDURE:

3.1 Vortex the 50 ml tube containing the bacterial pellet (from step 1.7) and transfer the contents to a new 1.5 ml tube. Note: There is no problem if a small amount of supernatant remains in the tube.

3.2 Add 300 µl of sterile water to the 50 ml tube, vortex, and transfer the content to the same 1.5 ml tube. Repeat the procedure.

3.3 Centrifuge the 1.5 ml tubes for 2 min at 12,000 g. Discard supernatant.

3.4 Wash the pellet with 600 µl of water. Centrifuge for 1 or 2 min at 12,000 g. Discard supernatant. Proceed with DNA extraction (Blood & Tissue Qiagen kit).

3.5 Resuspend the pellet in 180 µl Buffer ATL.

3.6 Add 20 µl Proteinase K. Mix thoroughly by vortexing, and incubate at 56°C, 500 rpm for 2 hours. Mix thoroughly and vortex each 30 minutes during incubation to disperse the sample.

3.7 Premix 200 µl Buffer AL and 200 µl ethanol (96–100%). Add 400 µl to the sample, and mix

thoroughly by vortexing.

3.8 It is essential that the sample, Buffer AL, and ethanol are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. A white precipitate may form on addition of Buffer AL and ethanol. This precipitate does not interfere with the DNeasy procedure.

3.9 Pipet the mixture from step 4 (including any precipitate) into the DNeasy Mini spin column placed in a 2 ml collection tube (provided). Centrifuge at 15,000 x g for 1 min. Discard flow-through and collection tube.

3.10 Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW1, and centrifuge for 1 min at 15,000 x g. Discard flow-through and collection tube.

3.11 Place the DNeasy Mini spin column in a new 2 ml collection tube (provided), add 500 µl Buffer AW2, and centrifuge for 3 min at 15,000 x g to dry the DNeasy membrane. Discard flow-through and collection tube.

3.12 Note: It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensures that no residual ethanol will be carried over during elution. Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this could result in ethanol carryover. If ethanol carryover occurs, empty the collection tube, then reuse it in another centrifugation for 1 min at 15,000 x g.

3.13 Place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipet 200 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 15,000 x g to elute.

3.14 Elution with 100 µl (instead of 200 µl) increases the final DNA concentration in the eluate, but decreases the overall DNA yield.

3.15 For maximum DNA yield, repeat elution i.e. place the DNeasy Mini spin column in a clean 1.5 ml or 2 ml microcentrifuge tube, and pipet the eluate (200 µl) onto the DNeasy membrane. Incubate at room temperature for 1 min, and then centrifuge for 1 min at 15,000 x g to elute.

4- Quantify DNA with a spectrophotometer (e.g. Nanodrop) -optional:

Standardize the DNA concentration to 20 ng/µl.

5- Run PCR

The standard assay is a SYBR-Green qPCR from Pal et al. (2019), but a supplemental conventional PCR assay from Thapa et al. (2012) can be added (not required) – Section 6. The SYBR-Green assay will produce an amplicon if *Pantoea stewartii* subsp. *stewartii* DNA is present. If absent, no signal will be seen.

The conventional PCR assay will produce an amplicon with either subspecies, a 375-bp band from *Pss* and a 193-bp band from *Psi.*

SYBR-Green PCR (Pal et al, 2019).

Primers -SYBR

Target	**Name**	**Sequence (5'-3')**	**Product size (bp)**
P. s. subsp. *stewartii*	*Cps AB* 2313 F	5'-AGA AAA CGC TGA TGC CAG AC-3'	256
P. s. subsp. *stewartii*	*Cps* R	5'- ACT ATC CTG ACT CAGGCA CT-3'	

5.1. Prepare SYBR PCR Mix (table 1).

5.2. Add 8 µl master mix per well to a 96-well PCR plate.

5.3. Add 2 µl of DNA template to duplicate wells.

5.4. Include: *Pss* positive control (PC1), a negative control (NC: *P. s.* subsp. *indologenes*) and a non-template control (NTC: dd-H_2O).

5.5. Seal the plate.

5.6. Perform the SYBR PCR and Melt Curve according to the protocol below (table 2).

Table 1. SYBR green real time Master Mix

Reagent	**µl /10 µl Reaction**
PowerUp SYBR Green Master Mix 2X	5 µl
10 µmol/L cpsAB2313F	0.4 µl
10 µmol/L cpsR	0.4 µl
PCR-grade water	2.2 µl
Template (20 ng/µl of purified genomic DNA)	2 µl
Reaction volume	**10 µl**

Table 2. SYBR PCR conditions

Cycles	Temperature	Duration
Hold	50°C	2 min
Hold	95°C	2 min
40 cycles	95°C	15 seconds
	60°C	15 seconds
	72°C	1 min

Melt Curve Analysis

SYBR green is a double-stranded DNA binding dye used to quantify amplicon amount during PCR by tracking overall fluorescence emission. After 40 cycles of amplification, a melt curve is generated by increasing the temperature from 60°C to 95°C in 0.5C increments. As the dsDNA is denatured (pulls apart), the fluorescence decreases. The change of fluorescence intensity displays as distinct peaks corresponding to the thermal melting point of each product. The melt curve distinguishes specific targets from other products such as primer- dimers or non-specific product amplification. **The melt temperature peak of *Pantoea stewartii* subsp. s*tewartii-specific* amplicons varies between 78**°C **– 82.5**°C **depending on the master mix and qPCR thermocycler used.** All amplicons should have a melt peak that aligns with the positive control.

Bio-Rad system: Run melt-curve from 65°C to 95°C in increments of 0.5°C
Applied Biosystems instruments:

Step	Ramp rate	Temp	Time
1	1.6°C/second	95°C	15 sec
2	1.6°C/second	60°C	1 min
3 (Dissociation)	0.15°C/second	95°C	15 sec

Melt curve ramp increment: Continuous

RESULTS:

Report qualitative (positive/negative) and quantitative (CT values) results for each subsample. A cut-off value is preferably set Ct < 32.

6- Conventional PCR (Thapa et al., 2012, DOI 10.1007/s13313-012-0123-9).

Conventional PCR is useful for distinguishing *P. stewartii* subsp. *stewartii* from *P.s.* subsp. *indologenes* using the primers *CpsF/CpsR.* The reverse primer (*CpsR*) is identical to *CpsR* used for the SyBR-Green assay but the forward primer (*CpsF*) is moved upstream toward the 5' end. The primers produce different PCR products for Pss and *Psi*, with a size difference that can be viewed on an agarose gel, a 375-bp band from *Pss* and a 193-bp band from *Psi.* Run the PCR products on a 1.5-2% agarose gel

containing SyBR-Safe or GelRed stain.

Primers

Target	Name	Sequence (5'-3')	Product size
P.s. subsp. *stewartii*	*Cps F*	5'- AAG GTG CCA GCC TCT CTC TG-3'	375 bp
P.s. subsp. *stewartii*	*Cps* R	5'- ACT ATC CTG ACT CAG GCA CT-3'	

Target	Name	Sequence (5'-3')	Product size
P.s. subsp. *indologenes*	*Cps F*	5'- AAG GTG CCA GCC TCT CTC TG-3'	193 bp
P.s. subsp. *indologenes*	*Cps* R	5'- ACT ATC CTG ACT CAG GCA CT-3'	

Conventional PCR conditions

Reagent	µl /15 µl Reaction
Go Taq Green Master Mix 2X (Promega)	7.5 µl
10 µmol/L cpsF	0.5 µl
10 µmol/L cpsR	0.5 µl
PCR-grade water	4.5 µl
Template (20 ng/µl of purified genomic DNA)	2 µl
Total reaction volume	15 µl

Cycles	Temperature	Duration
Hold	95°C	3 min
40 cycles	95°C	20 seconds
	60°C	30 seconds
	72°C	1 min

ANNEX A

SDS 10% Stock Solution
Add 10 g sodium dodecyl sulfate powder to 80 mL of MilliQ water. Heat to 68℃ for complete dissolution, then adjust to 100 mL with MilliQ water. The solution can be stored at room temperature for months. If precipitation occurs, re-heat the solution at 68℃ for 10 minutes. Do not autoclave or refrigerate, as they will cause precipitation.

CTAB/NaCl solution (10% CTAB in 0.7 M NaCl)
Dissolve 4.1 g NaCl in 80 ml water and slowly add 10 g CTAB (hexadecyltrimethyl ammonium bromide) while heating and stirring. If necessary, heat to 65℃ to dissolve. Adjust final volume to 100 ml.

5M NaCl
Dissolve 292.2 g sodium chloride in 1000 mL deionized water,

BUFFERS:

General Extraction Buffer (pH 7.4)

Sodium sulfite (anhydrous): 1.3 g

Polyvinylpyrrolidone (PVP) mol. wt. 24,000-40,000: 20.0 g

Sodium azide: 0.2 g

Egg albumin (Ovalbumin Grade II): 2.0 g

Tween-20: 20.0 g

Dissolve in 1000 ml of 1X PBST

Store at 4°C

PBST Buffer (1X, pH 7.4)

Sodium Chloride: 8.0 g

Sodium phosphate, dibasic (anhydrous): 1.15 g

Potassium phosphate, monobasic (anhydrous): 0.2 g

Potassium chloride: 0.2 g

Tween-20: 0.5 g

Dissolve in 1000 ml of distilled water

Substrate Buffer (pH 9.8)

Diethanolamine: 97 ml

Hydrochloric acid (32%): 15 ml

Add water to make to 1 liter.

Adjust pH if necessary with HCl.

REFERENCES:

Lamka, G. L., Hill, J. H., McGee, D.C. and Braun, E.J. 1991. Development of an immunosorbent assay for seed-borne Erwinia stewartii. Phytopathology 81:839-846.

Pal, N., Block, C. C., & Gardner, C. A. C. 2019. A real-time PCR differentiating *Pantoea stewartii* subsp. *stewartii* from *P. stewartii* subsp. *indologenes* in corn seed. Plant Dis. https://doi.org/10.1094/PDIS-06- 18-0936-RE.

Thapa, S. P., Park, D. H., Wilson, C., Hur, J. H., and Lim, C. K. 2012. Multiplex PCR assay for the detection of *Pantoea stewartii* subsp. *stewartii* using species-specific genetic markers. Australas. Plant Pathol. 41:559-564.