Conventional polymerase chain reaction (PCR) assay for detection and characterization of bacterial leaf blight in wheat applied by ICARDA's GHU

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DESCRIPTION:

Wheat is one of the most important crops for global food security and represents a main source of food and income for millions of smallholder farmers worldwide. *Pseudomonas syringae* pv. *syringae* (*Pss*) is the causal agent of bacterial leaf blight or leaf necrosis disease with specific symptoms on wheat leaves and spikes and in good conditions it can reach to the seeds to be seed borne disease. Yield losses caused by *Pss* can reach up to 50% or more depending on the crop, time of infection, and region. Therefore, developing and implementing effective management strategies for bacterial diseases is very important to reduce yield and quality loss. However, accurate detection of diseases is the first essential step for effective management strategies for control of this disease. Molecular is efficient tool to accurately detect and monitor the occurrence and development of crop diseases. This report describes a validated conventional polymerase chain reaction (PCR) assay for Pss detection and characterization applied by ICARDA's GHU.

Keywords: *Bacterial disease, seed-borne, PCR, plant health, seed testing, plant protection, food security.*

MATERIALS:

- A known strain of *Pseudomonas syringae* pv. *syringae*.
- Petri dishes plates (90 mm) of KBC medium.
- Eppendorf tubes (2 ml), containing sterile Nutrient broth (NB) medium.
- 70% ethanol for disinfection of surfaces and equipment.
- Incubator: operating at 28±2°C.
- Shaker incubator: operating at 28°C.
- Balance: capable of weighing to the nearest 0.001 g.
- pH meter: capable of being read to the nearest 0.01 pH unit.
- UV light (365 nm) to check fluorescence.
- Pipettes: capable of pipetting to the nearest 0.001 ml.
- Eppendorf tubes: 2 and 1.5 ml.
- Specific PCR Primers.
- Thermal cycler.
- Agarose electrophoresis equipment.
- Orbital shaker.
- Sterile pipette tips.

METHODS:

1. Bacteria extraction and identification (adapted to Schaad, 1982; Schaad *et al.,* 2001)

1.1. Isolation

- Disinfect the leave surface by washing in sodium hypochlorite 0.5% followed by washing by sterile water.
- Place the leaves on sterile filter paper inside petri dishes 90 mm diameter and incubate the leaves in incubator at 28°C overnight.
- Take from the bacteria ooze and spread over the surface of KBC plates by dilution technique.
- Incubate inverted plates 28±2°C and examine after 2-3 days.

1.2. Examination of the plates

- Examine KBC plates after 2-3 days, the colonies of *Pss* are creamy and half-translucent.

- Check the colonies under UV light (365 mm) and mark the blue florescent colonies (Figure 1).
- Pick the suspect colonies and subculture on KBC plates in dilution technique.
- Incubate inverted plates 28±2°C and examine after 2-3 days.

1.3. Identification of suspect colonies

- Examine KBC plates after 2-3 days.
- Recheck under UV light (365 mm).
- Identify suspect colonies sub-cultured with an oxidase test by putting a drop of 1% aqueous N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride solution on a filter paper. Add quickly a smear from a suspect bacterial colony on the filter paper and make a bacterial emulsion. *Pss* colonies are oxidase negative.
- Record results for each sub-cultured colony.
- All oxidase-negative, typical fluorescent colonies on KBC and all oxidase-negative colonies are considered suspect colonies.
- Confirm the identity of all the suspect colonies by polymerase chain reaction (PCR).

2. Molecular characterization

2.1. DNA extraction

- Make a slightly turbid cell suspension (OD_{600} nm approximately 0.1) in 1.0 ml of NB medium from the suspect cultures.
- Incubate in shaker incubator at 28±2°C to get OD_{600} nm=0.1 (usually 1 day).
- Centrifuge at 10,000 rpm for 15 min and discard the supernatant.
- Wash the sediment with 1 ml of sterile water and centrifuge again at 10,000 rpm for 15 min.
- Discard the supernatant and proceed to DNA extraction.
- Extract the DNA using DNeasy Plant Mini Kit (Qiagen, Cat. No. 69104) or any available DNA extraction kit.
- In addition to the DNA of tested samples, a positive control (DNA for identified culture) and a negative control (DNase free water) should be used as references.

2.2. Polymerase chain reaction (PCR)

- Prepare PCR reaction mixture using any PCR kit available (Table 1) using the following primer pairs (Manceau & Horvais, 1997):

  PSF: 5'-AGCCGTAGGGGAACCTGCGG-3'
  PSR: 5'-TGACTGCCAAGGCATCCACC-3'
- Carry out the PCR reactions in 0.2 ml thin-walled PCR tubes in a final volume of 10 µl (9 µl reaction mixture + 1 µl bacterial DNA) (Table 1).
- PCR profile: an initial denaturation of 95°C for 1 min, followed by 35 cycles of (95°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec) and a final extension of 72°C for 5 min.
- Fractionate 10 µl of the PCR products by gel electrophoresis for 1 h at 120 V on a 1.5% agarose gel in 0.5x Tris borate EDTA (TBE buffer) stained with RedSafe™ Nucleic Acid Staining Solution (20,000x) (Cat. No. 21141, iNtRON, South Korea), at final concentration of 5%. Include a 100 bp ladder (Solis BioDyne, Cat No. 07-11-0000S).
- Analyze the amplification products for a *Pss* specific product of 582 bp (Figure 2).

Table 1. Reaction mixture for PCR

PCR Reaction Mix*	Volume per tube (1x) µl
5X PCR buffer	2.0
Reverse primer (PSR) (10 pmol)	0.5
Forward primer (PSF) (10 pmol)	0.5
Taq DNA Polymerase	0.1
dH_2O	5.9
DNA template	1.0
Total	**10 µl**

***** *PCR Kit used: MyTaq™ Red DNA Polymerase from-Bioline-UK, Cat No# BIO-21108*

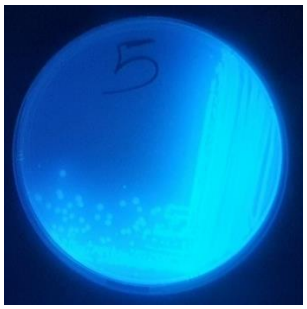

Figure 1. *Pseudomonas syringae* colonies on KB medium. Culture plate showing fluorescent pigment production under UV-transilluminator at 365 nm.

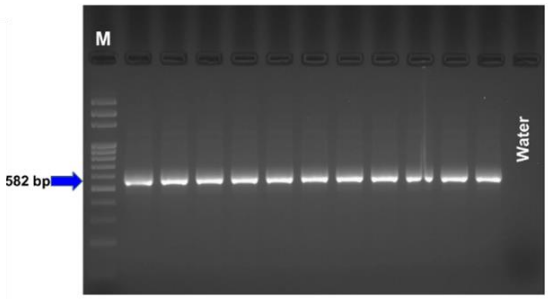

Figure 2. Detection of *Pseudomonas syringae* pv. *syringae* by direct PCR using specific primers PSF and PSR. M= DNA ladder VC100 bp Plus.

MEDIA AND SOLUTIONS:

KBC medium (adapted to Mohan & Schaad 1987)

- Proteose peptone (Difco No.3/Oxoid L46) 20.0 g/l
- K_2HPO_4 1.5 g/l
- $MgSO_4$. $7H_2O$ 1.5 g/l
- Agar 16 g/l
- Glycerol 10 ml/l
- Distilled water 900 ml
- Autoclave the solution and cool to 45°C. Then add the following ingredients aseptically:
    - Boric acid (0.5% aqueous solution) 100 ml autoclaved separately.
    - Cephalexin (stock solution of 10 mg/ml 8 ml distilled water).
    - Cycloheximide (stock solution of 25 mg/ml 8 ml 75% distilled water).

Nutrient broth (NB)

- Nutrient broth (Sigma-Aldrich) 23 g/l.
- Autoclave at 115°C for 20 min.

Note: *All prepared media plates should be inserted in polythene bags and stored at 4–8°C. Prepared plates can be stored for several months provided they do not dry out.*

10x TBE (1 liter)

- Dissolve 108 g Tris and 55 g Boric acid in 900 ml distilled water.
- Add 40 ml 0.5 M Na_2EDTA (pH 8.0) (alternatively use 9.3 g Na_2EDTA)
- Adjust volume to 1 Liter.
- Store at room temperature.

Prepare with DNase-free H_2O. Dilute 100 ml to 1 L to make gel running buffer.

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