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| Approved by | Officer In Charge  | <i>Approved</i> | 2 <sup>nd</sup> February 2023 |

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Required by: 1<sup>st</sup> February 2024

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| Approved by | Officer In Charge |           |      |

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Required by: 1<sup>st</sup> February 2027

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## 1.0 Purpose

1.1 This procedure explains steps followed during molecular detection of *Ralstonia solanaceum* *Ralstonia solanaceum*, *Ralstonia pseudosolanaceum* and *Ralstonia syzygii*, i.e. Phylotype/ sequevar strain in the *Ralstonia solanaceum* in potato tubers and stems.

## 2.0 Scope

2.1 This procedure covers sampling DNA extraction from samples, master mix preparation, PCR assays and results analysis by qPCR and conventional PCR techniques.

### 1. Definitions

DNA-deoxyribonucleic acid

NFW-Nuclease Free Water

RS-*Ralstonia Solanaceum*

|                                                                                          |                                                                 |                                                                                     |
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3.1 PCR-Polymerase chain reaction

3.2 qPCR-quantative Polymerase chain reaction

3.3 Mastermix-components for PCR reaction

## **2. Equipments**

4.1 Real-time PCR

4.2 Centrifuges

4.3 Fume chamber

4.4 Water bath

4.5 Forceps and Knives,

4.6 Grinding apparatus

4.7 96 well PCR plates

4.8 Thermocycler

4.9 Complete Electrophoresis unit ( Gel, Buffers, Trough )

4.10 Pipettes( 1000uL, 200ul, 10ul).

4.11 UV transuliminator

4.12 Refrigerator

4.13 Freezer

4.14 Microwave

|                                                                                          |                                                                 |                                                                                     |
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### 3. Responsibilities

#### 6.1.1 Head Molecular Laboratory

Ensures availability of reagents and apparatus for use

6.1.2 Ensure all reagents are valid for use.

6.1.3 Ensure machines and apparatus are all calibrated and operational.

6.1.4 Ensure appropriate protective and safety measures are in place.

#### 6.1.1 Method Principles

6.1.1.1 The Real-time PCR (qPCR) for *Ralstonia solanacearum* is performed using Rs primers and Rs P probes of Weller et al. (2000). This set of primers detects all known strains of *R. solanacearum* including Indonesian isolates of both the banana blood disease bacterium (phylotype IV) and *R. syzygii* from clove. B2 set of primers and probes are used to detect *R. solanacearum* Race 3 Biovar 2 in potato tubers and stems.

6.1.1.2 The conventional PCR uses OL1 and Y2 set of primers to detect *R. solanacearum* (Fegan et al., 1998).

### 6.2 Procedure for Extraction of DNA from potato tubers /stems

#### Sampling and sample preparation

- Potato tubers will be washed with water to remove soil and debris. The tubers will be sterilized using 1% NaOCl for 30 minutes and 70% ethanol
- For certification 400 tubers will be sampled randomly. These tubers will be divided into 10 sub samples of 40 tubers each. Each sub sample will be analyzed as a unit sample. Records of sampling data will be maintained
- For stem samples, obtain 3cm cuts of the stems for each sample and put in sampling bag.
- For potato tubers Extract the cores/eyes/vascular rings of the tubers put in a sampling polythene bag

|                                                                                          |                                                                 |                                                                                     |
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### 6.2.1 Isolation of DNA

### 6.2.2 Add 2ml of RNase free water and grind.

- Transfer the sap in a microfuge and centrifuge extract for 1min at 1000rpm to sediment plant tissue. Take 0.5ml of supernatant and place into a new Eppendorf tube; then centrifuge for 10min at 12000rpm.
- Discard the supernatant and clean the pellet by adding 1000ul of sterile double-distilled water, vortex and again centrifuge for 10min at 11000rpm.
- Discard 800ul of the supernatant.
- The pellet and remaining supernatant (200ul) is used for DNA extraction.
- Place the pellet and the supernatant (200 ul) in a heating block/water bath (rupture the bacterial cell and get the DNA) for 4mins at 96°C, cool for 10min on ice and centrifuge for 3min at 1250rpm.
- Save 150ul of supernatant and discard the pellet (pellet contains the DNA + inhibitors, while the supernatant has the DNA dissolved in it).
- Keep the DNA (supernatant) at -20°C prior to use.

**N/B:** - In case one has a culture, you start from step 6.2.2- extraction.

### 6.3 Real-Time PCR test-master mix preparation

#### Primers:

RS-I-F5' –GCA TGC CTT ACA CAT GCA AGT C – 3'

RS-II-R 5' –GCC ACG TTC CGA TGT ATT ACT CA -3'

#### Probes:

RS-P 5' - [FAM] –AGC TTG CTA CCT GCC GGC GAG TG – [TAMRA] -3'

B<sub>2</sub> –II-R AAT CAC ATG CAA TTC GCC TAC G

B<sub>2</sub> –I- F TGG CGC ACT GCA CTC AAC

B<sub>2</sub> P VIC TTC AAG CCG AAC ACC TGC TGC AAG [TAMRA]

|                                                      |                                                                     |                                                                                     |
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#### 6.4 *Master Mix template table for Real-time PCR*

- Master Mix should be prepared in sterile cabinet and the ingredients should be placed in ice
- Always allow reagents to thaw before preparation
- Prepare master mix by mixing the reagents as per the table below and dispense in the plate or PCR tubes accordingly

| Reagent                     | Quantity per reaction (ul) | Final concentration |
|-----------------------------|----------------------------|---------------------|
| Sterile NFW                 | 12.875                     | -                   |
| 10X PCR Buffer              | 2.5                        | 1x                  |
| Mgcl <sub>2</sub> (25 mM)   | 3.5                        | 3.5mM               |
| dNTP mix (2.5mM)            | 2.0                        | 200uM               |
| RS-I-Forward primer (10uM)  | 0.75                       | 300nM               |
| RS-II-Reverse primer (10uM) | 0.75                       | 300nM               |
| Probe RS (5uM)              | 0.5                        | 100nM               |
| Taq polymerase (5U/ul)      | 0.125                      | 0.63U               |
| Sample (DNA)                | 2ul                        | -                   |
| <b>Total</b>                | <b>25ul</b>                | -                   |

- Plate layout should be prepared and template added as the plan
- Seal the plate with adhesive cover and place in the thermal block in PCR machine

|                                                                                          |                                                                 |                                                                                     |
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#### 6.4.1 Run the PCR using following temperature protocol:

- 1 Cycle of: 10 minutes at 95 °C.
- 40 Cycles of: 15 Seconds at 95 °C and.
- **60 Seconds at 60 °C (The PCR machine collects the Fluorescence data between the annealing and elongation of DNA -).**Convectional PCR set-up

#### Primers

- Forward primer OLI-1 5'-GGG GGT AGC TTG CTA CCT GCC-3'
- Reverse primer Y-2 5'-CCC ACT GCT GCC TCC CGT AGG AGT-3'
- Expected amplicon size from *Ralstonia solanacearum* template DNA = 288 bp

#### 6.4.2 Convectional PCR reaction mix preparation

Master mix prepared as per below table and dispensed in PCR tubes

| Reagent                                                | Quantity per reaction | Final concentration            |
|--------------------------------------------------------|-----------------------|--------------------------------|
| Sterile ultra pure water                               | 17.65 µl              |                                |
| 10x PCR buffer <sup>1</sup> (15 mM MgCl <sub>2</sub> ) | 2.5 µl                | 1x (1.5 mM MgCl <sub>2</sub> ) |
| d-nTP mix (20mM)                                       | 0.25 µl               | 0.2 mM                         |
| Primer OLI-1 (20 µM)                                   | 1.25 µl               | 1µM                            |
| Primer Y-2 (20 µM)                                     | 1.25 µl               | 1µM                            |
| Taq polymerase (5U/µl) <sup>1</sup>                    | 0.1 µl                | 0.5 U                          |
| Total master mix                                       | 23 µl                 |                                |
| Template                                               | 2.0 µl                |                                |

- Template is added as per the plate plan and the tubes are tightly sealed
- Tubes are placed on the thermocycler block

#### 6.4.3 PCR reaction conditions

Run the following programme:

|                                                                                          |                                                                 |                                                                                     |
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1 cycle of: (i) 2 minutes at 96 °C (denaturation of template DNA)

35 cycles of: (ii) 20 seconds at 94 °C (denaturation of template DNA)

(iii) 20 seconds at 68 °C (annealing of primers)

(iv) 30 seconds at 72 °C (extension of copy)

1 cycle of : (v) 10 minutes at 72 °C (final extension)

(vi) hold at 4 °C (optional – only when you intend to run the gel electrophoresis at a later time.

## 7.0 Gel Electrophoresis

### Preparation of gels

- Weigh 1.5 g of agarose gels and dissolve in 125 mls TBE buffer
- Heat in the microwave to boil
- Allow to cool and add staining gel and carefully pour into the casting tray and leave to solidify
- ***Remove the combs and load 10 µl of amplified DNA reaction mixture from each sample***
- Connect the electrophoresis unit to power supply and leave to run for 100v for 1 hr
- Observe the gel under UV trans illuminator and visualize the bands to check expected fragment size

## 8.0 Results interpretation

- 8.1 In qPCR, amplification of the DNA strand as a sigmoid curve indicates the presence of the pathogen under test. Negative results are depicted by lack of a good sigmoid curve.
- 8.2 In convectional PCR, positive results are indicated by the presence of bands of 288bp on agarose gel viewed under UV transuliminator.
- 8.3 The test should be run with the following as control parameters
1. Positive sample

|                                                                                          |                                                                 |                                                                                     |
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2. Extraction blank
3. Non template
4. Molecular biology water

8.4 The results are recorded as per the attached plate map (Annex 1)

8.5 Results are reported as per SOP GE/10/Reporting test results.

#### **9.0 Storage of reference standards and reference materials.**

9.1 Positive controls are sourced from positively identified samples and validated for use.

9.2 All apparatus and working areas are supposed to be disinfected using 0.5n% sodium hypochlorite and 70% ethanol before and after work.

#### **10.0 References**

1. **Weller, S.A., Elphinstone, J.G., Smith, N.C., Boonham, N., and Stead, D.** 2000. Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time fluorogenic PCR (TaqMan) assay. *Applied and Environmental Microbiology* 66: 2853-2858.
2. **Fegan, M., Holoway, G., Hayward, A.C., and Timmis, J.** 1998. Development of a diagnostic test based on the polymerase chain reaction (PCR) to identify strains of *R. solanacearum* exhibiting the biovar 2 genotype. *In: Bacterial Wilt Disease: Molecular and Ecological Aspects.* P. Prior, C. Allen, and J. Elphinstone (Eds.). Springer-Verlag, Berlin. Pp. 34-43.

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### 11.0 Records

11.1 Sample reception book

11.2 Raw data book

11.3 Plate plan

11.4 Laboratory reports

### 12.0 Revision list

| Current Version number | Superceded version number | Revision summary                                                | Contributors                                     |
|------------------------|---------------------------|-----------------------------------------------------------------|--------------------------------------------------|
| 01                     | None                      | New                                                             | J.Waithera , G. Ngundo,<br>J.Wanjiku, S.Gachamba |
| 02                     | 01                        | Numbering of contents and editing to suit practice.             | J.Waithera , G. Ngundo,<br>J.Wanjiku, S.Gachamba |
| 03                     | 02                        | Inclusion of <i>Ralstonia syzigii</i> as per new classification | J.Waithera , S.Gachamba                          |

