Recombinase Polymerase Amplification (RPA) for detection of *Phytophthora* genus and species-specific: *P. sojae – P. sansomeana*

I. Reagents, primers and probes

A. Reagents

- Mesh bags (Agdia, Cat No. 00930)
- GEB2 Buffer (Agdia, Cat No. 00130)
- Tissue homogenizing tool (Agdia, Cat No. ACC 00900)
- Microcentrifuge 1.5 mL tubes
- Two suppliers available for RPA kit (includes rehydration buffer and magnesium):
    - AmplifyRP XRT kit (Agdia, XCS 99200/0048)
    - TwistAmp Exo (TwistDx, Cat No. TAEXO02KIT; http://www.twistdx.co.uk/)
- Molecular grade water
- Microcentrifuge amber-colored tubes
- PCR 8-tube strip
- Detection unit:
    - Smart-dart (www.diagenetix.com)
    - TwistA (www.twistdx.co.uk) or T-16 (www.agdia.com)

B. Primers

Primer	Sequence (5' - 3')	Length (bp)
Phytophthora genus-specific		
TrnM-F	ATGTAGTTTAATGGTAGAGCGTGGGAATC	29
TrnM-R	GAACCTACATCTTCAGATTATGAGCCTGATAAG	33
Phytophthora species-specific		
Atp9-F	CCTTCTTTACAACAAGAATTAATGAGAACCGCTAT	35
Psojae-nad9-R	AAATCTATCTTTGTATTCATATATCAAT	28
Psan-nad9-R	TTAGTAGTTAGTACTAATATAACAAAAATATAATA	35
Plant Internal Control		
Cox1-IPC-F	CATGCGTGGACCTGGAATGACTATGCATAGA	31
Cox1-IPC-R	GGTTGTATTAAAGTTTCGATCGGTTAATAACA	32

C. Probes

Probes*	Sequence (5' - 3')	Length (bp)
Phytophthora genus-specific		
TrnM-P (Probe)	TAGAGCGTGGGAATCATAATCCTAATGTTG [**FAM-dT**] A [**THF**] G [**BHQ1-dT**] TCAAATCCTACCATCAT [**3'-C3SPACER**]	51
Phytophthora species-specific		
Atp9-P (probe)	TTGCTTTATTYTGTTTAATGATGGCWTTY [**FAM-dT**] T [**THF**] A [**BHQ1-dT**] YTTATTTGCTTTTT [**3'-C3SPACER**]	47
Plant Internal Control		
Cox1-IPC-P (probe)	GGTCCGTTCTAGTGACAGCATTCCYACTTTTATTA [**TAM-dT**] C [**THF**] C [**BHQ2-dT**] YCCGGTACTGGC [**3'-C3SPACER**]	51

* Probes are ordered from Biosearch technologies, Inc. (https://www.biosearchtech.com/) as custom oligos.

II. Sample processing

1. Collect root tissue with symptoms of root rot, taking samples if possible from the margin between necrotic tissue and healthy tissue.
    a. If possible wash the root system using tap water to remove soil and rhizosphere adhered to the tissue. Pat the root tissue dry on the towel papers.

2. GEB2 buffer should be prepared on the same day of sample processing. Prepare enough buffer for your samples plus two extra samples to account for error. For one sample, add 0.279 g of GEB2 powder into 5 mL of sterile distilled water.

3. Weigh 500 mg of tissue and place it in the mesh bag and add 5 mL of GEB2 buffer into the mesh bag.

4. Fold the top of the mesh bag, and grind the tissue in the mesh bag with the homogenizing tool. Be careful since samples can perforate the bag and cause cross-contamination.

5. Crude extract can be used directly or transfer an aliquot of the crude extract into a microcentrifuge tube and store at -20ºC.

III. Preparation probes and primers

1. Centrifuge briefly the tubes with primers and probes for 20 secs at 10,000 rpm before opening the tubes.

2. **100 µM stock solutions:** dissolve primers and probes by adding molecular grade water for a final concentration of 100 µM. Store probes in amber-colored tubes at -20ºC or -80ºC if possible.

3. **10 µM working solutions:** Prepare working solutions by making a ten-fold dilution from the stock solutions using molecular grade water and store 100 µL aliquots at -20ºC or -80ºC if possible. Use amber-colored tubes for probes.
    a. For example, to make 500 µL of primer working stock, dilute 50 µL of the primer/probe and dissolve in 450 µL of molecular grade water.

4. **1 µM working stocks:** Primers for *Phytophthora* genus-specific (**TrnM-F)** and *Phytophthora* species-specific (**Atp9-F)** are required to be a 1 µM working stocks to avoid pipetting errors, hence a 100-fold dilution from stock solutions should be made using molecular grade water. Store 100 µL aliquots at -20ºC or -80ºC, using amber-colored tubes for probes.
    a. For example, to make 500 µL of primer working stock, dilute 5 µL of plant internal control primer and dissolve in 495 µL of molecular grade water.

IV. Master mix preparation

1. Thaw primers and probe. Rehydration buffer and magnesium acetate can be stored at room temperature. Vortex briefly for 10 sec at setting 7, then spin for 10 sec in a microspin centrifuge. Place all the reagents on ice.

2. Prepare the master mix in a clean area; make the master mix volume using the table below depending on the target. Keep master mix on ice before use.

    a. All the volumes for the reaction master mix were calculated based on the AmplifyRP XRT kits from Agdia. If the TwistAmp Exo kits are being used, double the volumes, since pellets from this company are designed for 50 µL reactions.

A. *Phytophthora* genus-specific RPA

Reagents	Initial concentration	RPA *Phytophthora* genus volume per reaction (µL)			
		1X	4.5X	8.5X	Check
Genus-specific					
TrnM-F	1 µM	0.50	2.3	4.3	
TrnM-R	10 µM	1.45	6.5	12.3	
TrnM-Probe	10 µM	0.35	1.6	3.0	
Plant Internal Control					
Cox1-IPC-F	10 µM	0.63	2.8	5.3	
Cox1-IPC-R	10 µM	0.63	2.8	5.3	
Cox1-IPC-Probe	10 µM	0.30	1.4	2.6	
Rehydration buffer		14.75	66.4	125.4	
Water		4.15	18.7	35.3	
Crude Plant Extract/DNA		1.00	4.5	8.5	
Total Volume*		**25 µL**	**112.5 µL**	**212.5 µL**	

* This volume is after adding the magnesium acetate, but this is added just before the start of the reaction.

B. *Phytophthora sojae* species-specific RPA

Reagents	Initial concentration	RPA *Phytophthora sojae* volume per reaction (µL)			
		1X	4.5X	8.5X	Check
Species-specific					
Atp9-F	1 µM	0.3	1.1	2.1	
Psojae-nad9-R	10 µM	2.1	9.3	17.6	
Atp9-Probe	10 µM	0.5	2.0	3.8	
Rehydration buffer		14.8	66.4	125.4	
Water		5.30	23.9	45.1	
Crude Plant Extract/DNA		1.0	4.5	8.5	
Total Volume*		**25 µL**	**112.5 µL**	**212.5 µL**	

* This volume is after adding the magnesium acetate, but this is added just before the start of the reaction.

C. *Phytophthora sansomeana* species-specific RPA

Reagents	Initial concentration	RPA *Phytophthora sansomeana* volume per reaction (µL)			
		1X	4.5X	8.5X	Check
Species-specific					
Atp9-F	1 µM	0.5	2.3	4.3	
Psan-nad9-R	10 µM	2.1	9.2	17.4	
Atp9-Probe	10 µM	0.5	2.0	3.8	
Rehydration buffer		14.8	66.4	125.4	
Water		5.00	22.5	42.5	
Crude Plant Extract/DNA		1.0	4.5	8.5	
Total Volume*		**25 µL**	**112.5 µL**	**212.5 µL**	

* This volume is after adding the magnesium acetate, but this is added just before the start of the reaction.

3. Mix the master mix by inverting tube up and down and spin it down in a microspin centrifuge for 10 sec. Then aliquot 22.75 µL into each well of a 250 µL 8-well PCR strip, maintain tubes or plates on ice.

4. Use the crude extract, if the extract was frozen, thaw it and mix well and let it settle for a few minutes. Take 1 µL of crude extract and add it into each well.

5. Transfer the 23.75 µL from the 8-well PCR strip into the AmplifyRP XRT strip containing the lyophilized pellets and mix to dissolve the pellet.

6. Pipette 1.25 µL of magnesium acetate into the cap of each well strip, carefully close the caps and centrifuge the strip using a small bench centrifuge, invert tubes 10 times and re-centrifuge.

    a. **It is important to note that the Mg initiates the amplification even under room temperature, so it is important to add the Mg at this point and also for consistent results to make sure the cap is secured in a way that prevents one tube from getting Mg before the others.**

7. Place the reactions on the platform; follow instructions below depending on the equipment available.

D. TwistA platform

1. Open the Twista software on the computer, change the time of the assay to 25 for XRT reactions and press the play button.

2. After 4 minutes, remove the tubes, invert 10 times and re-centrifuge and place back in the Twista.

3. Collect results, reaction times vary depending on initial template concentration.

4. For result interpretation, select the slope validation method and input 6 min and 25 min as the period to evaluate the reactions, 4 points within this range and slope of 30 mV/min, to determine positive samples.

E. Smart-Dart platform

Creation of a new method

The set-up of a new protocol has to be done before any reaction set-up or sample diagnosis, to establish the method for the *Phytophthora* RPA follow the instructions below.

- Open the Smart-Dart software on the tablet, and select "Options" on the lower part of the screen.
- A list will show up and select the option "Edit methods"
- Select the option "Add new method" and a new screen will appear with text boxes to input the reaction parameters.
- Insert the method name, in this case "Phytophthora RPA"
- In the "LAMP temperature" box input 39ºC and in the "LAMP reaction time (min)" input 25.
- For the options "Denature Temperature" and "Denature time" input 39ºC and 0 respectively.
- Finally, input 20 in the option "Fluorescence reading interval (sec)" and tap on confirm.
- The method should be listed now on the "Edit methods" screen and in the "Analysis Method" menu.

Reaction set-up

1. Incubate the reactions at 37ºC on a heat block for 3 min.

2. After 3 minutes, remove the tubes, invert 10 times and re-centrifuge, and place the tubes on the Smart-Dart.

3. Start the Smart-Dart software on the tablet, and establish a connection with the unit by selecting option "Connect Menu" and select the unit available.

4. Go to the option “Analysis Method” and select the method for the assay, in this case *Phytophthora* RPA.

    a. Before starting the reactions, create a new method if the option is not available (previous section).

5. Tap on the option “Real-Time LAMP” to start the reaction.

V. References

Miles, T. D., Martin, F. N. & Coffey, M. Development of rapid isothermal amplification assays for detection of Phytophthora species in plant tissue. Phytopathology (2014). doi:10.1094/PHYTO-05-14-0134-R

Piepenburg, O., Williams, C. H., Stemple, D. L. & Armes, N. A. DNA detection using recombination proteins. PLoS Biol 4, e204 (2006).