NATIONAL PLANT PROTECTION CENTRE
DEPARTMENT OF AGRICULTURE
MINISTRY OF AGRICULTURE AND LIVESTOCK

NPPC

Standard Operating Procedure (SoP) for Plant disease diagnosis (Laboratory method)

Pathology Program

2024

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Disclaimer: *The Protocols and Procedures are dynamic and subject to modification as per the availability of materials and facilities.*

Standard Operating Procedure (SoP) for plant disease diagnosis (Laboratory methods)

Pathology Laboratory
National Plant Protection Referral Laboratory
National Plant Protection Centre
Semtokha, Thimphu

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SoP-1: Diagnostic Protocol for Huanglongbing disease

'*Candidatus* Liberibacter' species

Disease

Huanglongbing (HLB) is the official common name. Other common names are citrus greening, yellow shoot and many more in earlier literatures.

Hosts

The disease affects *Citrus* and some genera in Rutaceae.

Taxonomy information

Name: '*Candidatus* Liberibacter africanus' Garnier et al. 2000; Synonym: '*Candidatus* Liberibacter africanum' Jagoueix et al., 1994.

Name: '*Candidatus* Liberibacter americanus' Texeira et al. 2005; Synonym: '*Candidatus* Liberibacter americanum' Jagoueix et al., 1994.

Name: '*Candidatus* Liberibacter asiaticus' Garnier et al. 2000; Synonym: '*Candidatus* Liberibacter asiaticum' Jagoueix et al., 1994. Occurs in

Taxonomic position

Domain – Eukaryota
Kingdom – Bacteria
Phylum – Proteobacteria
Class – Alpha-Proteobacteria
Order – Rhizobiales
Family – Phyllobacteriaceae
Genus – Liberibacter
Species – '*Candidatus* Liberibacter africanus'; '*Candidatus* Liberibacter americanus''*Candidatus* Liberibacter asiaticus';

Distribution

'*Candidatus* Liberibacter africanus' (CLaf) – present in Asia and Africa (Bové 2006).

'Candidatus Liberibacter americanus' (CLam) – present in Brazil, South America (Bové 2006).

'*Candidatus* Liberibacter asiaticus' (CLas) – present in Asia, Africa, Oceania, North and South America (Bové 2006).

Vectors

'*Candidatus* Liberibacter africanus' – vectored by *Trioza erytreae* (Bové 2006).

'Candidatus Liberibacter americanus' – vectored by *Diaphorina citri* (Bové 2006).

'*Candidatus* Liberibacter asiaticus' – vectored by *Diaphorina citri* (Bové 2006).

Detection

Symptoms

Symptoms of HLB are not specific and may be confused with nutrient deficiency and symptoms due to other factors like water logging, root or trunk rots, root or trunk boring insects etc.

Some typical symptoms associated with the HLB pathogens are yellow shoots and blotchy mottle symptoms (Figure 1.). Trees show yellow shoots due to irregular distribution of the bacterium in the infected tree. Leaves on infected branches will show blotchy mottle and some may show nutritional deficiencies e.g., zinc. Fruits may develop colour inversion where colouring start from the peduncle end in contrast to the styler end colouring first. Fruits may taste bitter.

Figure 1. Huanglongbing symptoms: (left) yellow shoots; (right) blotchy mottle on mandarin leaves (Photo: N.Om).

Detection using molecular methods

Electron microscopes and bioassays using indicator plants were common methods of detection before molecular methods were developed. In the recent years, molecular tools such as polymerase chain reaction (PCR) provide the most reliable and confirmatory tests for detecting HLB pathogens. Both conventional and real-time PCRs are applicable for detection of the HLB pathogens in both plant and vectors. Primers and probes are given in Table 1.

Sample collection

Plant sample

Methods are developed based on literatures as well personal experience.

Materials required for sampling:

- Plastic bags or zip lock bags
- Stapler and pins
- Pencil
- Labelling paper (made from A4)
- Knife or a leather punch (of ~6-8mm in diameter)
- 5 mL vials with screw caps
- 80% ethanol

- Bleach
- Cool box or locally available materials

Leaf samples

For symptomatic mature trees of about ≥ 10 years old if yellowing branches are present, symptomatic leaf samples should be collected from the yellowing branches. Two leaf samples and one bark per tree should be collected.

- Look for symptomatic branches e.g., yellow branches. Within the symptomatic branch, look for mottled leaves, yellow leaves, leaves with yellowing veins and/or corky veins, small upright leaves, leathery leaves, nutrient deficient leaves e.g., Zn deficiency.
- From each symptomatic branch collect at least 10-12 symptomatic leaves (with petioles) of about 10cm long per sample. Note: if leaves are of small size, then increase the number of leaves up to 15-20.
- From each tree, include one bark samples comprising of young twigs and main trunk (refer below for bark sampling).
- For asymptomatic trees, collect two leaf samples from around the canopy of the tree, and one bark sample.
- Do not collect samples from yellowing branch due to physical damage as in the case of trunk borer infestation or other causes.
- Place each sample in a plastic bag with labels (written with pencil on plain paper).

Bark samples

- Use a leather puncher or a sharp knife
- Take at least 6 pieces of bark per sample from a branch (~6-8mm in diameter if using puncher or length if using a knife) OR young twigs of about 10-15 cm with green barks. Collect at least 4-6 sections of young twigs per sample. If symptomatic branch is present, collect barks from such branch and also from the main trunk.
- For barks from the main trunk, collect around 6 pieces above the soil.
- Place the composite samples in a plastic bag and label.
- Clean the tools with bleach before using one the next tree (e.g., Robin fabric whitener): take about 150 mL bleach in a 250 mL container and immersing the entire tip which cuts through bark into the liquid, then wipe with clean cotton or tissue paper.
- Clean and oil the tools with sewing machine oil after use.

Vector/psyllid sample

Sampling adult psyllids:

Adults are present throughout the year if high population occurs during flush period.

- Tap branches onto a bowl or tray.
- Suck the falling adults using an insect aspirator (or pooter). Some bigger nymphs may also fall during tapping and the nymphs can be collected as well.

- Alternately, adults can be collected directly from the trees using a manual or battery-operated pooter.
- Collect at least 20-30 adults per site e.g., orchard or nursery.
- Transfer pysllids (both adults and nymphs collected by tapping) into tubes with 80% ethanol
- Label each tube: date, location name and GPS coordinates, host plant name/variety, psyllid species, psyllid stage.

Sampling nymphs:
Nymphs of *Diaphorina* species can be brushed off from the shoots or twigs and place in vials containing 80% ethanol.

Nymphs of the citrus green psyllid, *Cacopsylla heterogena* can be collected as follows:

- Nymphs develop inside the folds of young tender leaves call 'pouch galls'.
- Look for trees with pouch galls on young shoots.
- Collect the pouch galls that have nymphs inside (bigger nymphs are desirable, refer pictures). Place all the pouch galls with nymphs straight into a 5 mL tube and pour 80% ethanol.
- Keep samples collected from different trees separately and label accordingly if infection on individual tree is to be tested. Label each tube: date, tree number (as per the germplasm map), host plant name/variety.

Sample care and submission to NPPC

- If leaves are wet from rain or dew then wipe dry with tissue paper before packing in the plastic bags.
- Keep all samples cool after collection, preferably in the fridge at 4°C (not the freezer) till transportation.
- Prepare a sample list containing sample details according to the labels (for plant parts and psyllids).
- Dispatch samples to NPPC within 1-2 days of collection along with the list. Transport in cool boxes.

Labelling samples

- Labels should include: date of collection, location name & GPS coordinates, species and variety name for plant sample and in case of psyllid, species name and variety of the host plant where psyllids are collected from, and collector name.
- Labels can be hand written using pencil or typed (laserjet print).

Once sample reach the laboratory

- Open package in the extraction room.
- Verify samples according to the label and sample list.

- Record in the sample register and assign laboratory number.
- Store samples at 4°C during verification and extraction.
- Process DNA extraction.

DNA extraction

Although several methods of DNA extractions are available, in NPPC we use the CTAB method because it is a cheap method that can yield good quality DNA. Many commercial DNA extraction kits which provide more rapid extraction protocols are available from many manufactures e.g., Sigma, Bioline and Qiagen and can substitute the current method of CTAB. The CTAB protocol detailed below is applicable for both plant and psyllid samples. If using a DNA extraction kit, extraction procedures must follow the manufacture's instruction or with modifications if required. DNA extraction kits are more convenient and may be safer than using chloroform to separate organic soluble from DNA but they are expensive.

Some of the DNA extraction kits that can be used are:

- ISOLATE II Plant DNA kit and ISOLATE II Genomic DNA kit from Bioline (used for psyllids). Kits from BIOLINE are cheaper and give similar DNA quality as to using more expensive kits.
- For psyllids, REDExtract-N-Amp™ Tissue (or plant) PCR Kit from Sigma works very well and provides a rapid DNA extraction protocol for psyllids.
- DNeasy Plant Pro and Plant kits from Qiagen.

DNA extraction from plant samples using CTAB (modified Doyle and Doyle, 1990)

1. Label all tubes and plastic pouch before starting extraction: two sets of 2 mL tubes and one set of 1.5 mL tube. Turn on the water bath with the temperature set at 65°C.
2. Wash samples thoroughly (or if using barks: place the barks in a petri dish with distilled water, clean the barks by scrapping off all dead tissues on the outside very lightly with a scalpel, then rinse with distilled water two times). *Include an extraction control comprising of a disease free plant (same species if available if not any other plant).*
3. Blot dry with tissue paper/paper towel.
4. Using disposable blades, cut mid ribs (or cut the barks) and transfer (0.5 g) in the extraction pouch.
5. Add 3–3.5 mL of CTAB (add 20 µL of β-mercapto ethanol per 10 mL of CTAB just before use to each pouch. S
6. Grind the samples with the homogenizer at 3000 rpm until no tissue are visible. Homogenization can also be done using Fastprep instrument.
    a. If using FastPrep, grind plant tissue using liquid nitrogen before placing CTAB: place 0.5g tissue in 2mL tube (special tube with screw cap), place sterile 6 mm diameter beating beads, dip tubes in liquid nitrogen for 10 sec then place frozen tubes in the FastPrep instrument and secure well. Pulse following FastPrep protocol. Once tissue is ground to powder, add CTAB filling the tube to 1.9 mL. Then proceed to step 8 below.
7. Transfer all extracts into a 2 mL tube with a pastuer pipette.

8. Place all the tubes in a floater and incubate in the water bath for 30 min at 65°C (shake the tubes by inverting the tubes gently after 15 min).
9. Centrifuge for 5 min at 3000 rpm (~8000 x g) and 20°C.
10. Transfer 1 mL of the supernatant into another set of 2 mL tubes.
11. Add 1 mL of chloroform:Isoamyl alcohol (24/1) to each tube. Shake by inverting the tubes a few times.
12. Centrifuge at 20°C and 14000 rpm (~16100 x g) for 5 min.
13. Transfer ~0.850 to 0.9 mL (usually it is easier to transfer 850 µL without sucking the lower phase of the liquid) of the supernatant to 1.5 mL tube. Take care not to take in any chloroform from the bottom – if this happens, put back everything in the original tube and centrifuge again.
14. Add 0.54 mL (540 µL) of Isopropanol to each tube. (Isopropanol should be stored at −20°C).
15. Precipitate at −20°C for 1 hr if to be continued on the same day or at 4°C overnight if to be continued on the next day.
16. Centrifuge at 4°C and 14000 rpm for 20 min.
17. Discard the supernatant.
18. Re-suspend the pellet in 1mL of 70% ethanol.
19. Vortex slightly/mix by hand for few seconds by inverting.
20. Centrifuge at 20°C and 14000 rpm for 5 min.
21. Discard the supernatant.
22. Re-suspend the pellet in 95% ethanol.
23. Vortex slightly or hand mix by inverting.
24. Centrifuge at 20°C and 14000 rpm for 5 min.
25. Discard the supernatant. Carefully siphon off the remaining supernatant with a smaller calibrated pipette (20-200 µL). Discard tip for each sample.
26. Leave open the tubes for 30 min to 45 min on bench top.
27. Re-suspend the pellet in 80–100 µL of PCR water (or sterile distilled water) or in 1x TE buffer for long term storage or if transporting.
28. Store DNA at −20°C till further use.

DNA extraction from psyllids using CTAB

1. Although single adult psyllid has been used to test for HLB pathogen, in our protocol we use groups of five psyllids (adults or 4–5th instar nymphs) for DNA extraction. If fewer psyllids are available, then use whatever number (> 1) is available but decrease the DNA elution volume to 20 µL.
2. For psyllids preserved in ethanol, dry the psyllid completely to let the ethanol evaporate. This can be achieved by placing the psyllids on clean paper towel using a fine tip brush. Then pick the desired number e.g., five adult psyllids, into each 1.5 mL tubes. Leave the tube open (in the laminar hood) for 10 min before adding extraction buffer. *Include*

extraction control using a disease free psyllid if available or another insect that is not a vector of CLaf/CLam/CLas, e.g., house fly.

3. In each 1.5 mL tube containing psyllids, add 300 µL CTAB buffer and crush with a sterile plastic pestle.
4. Once the psyllids are macerated, incubate for 30 minutes at 65°C (shake the tubes by inverting the tubes gently after 15 min).
5. Centrifuge for 5 minutes at 3000 rpm (~8000 x g) at 20°C.
6. Transfer 150 µL of the supernatant into another set of 2 mL tubes.
7. Add 150 µL of chloroform: Isoamyl alcohol (24/1) to each tube. Shake by inverting the tubes a few times.
8. Centrifuge at 20°C and 14000 rpm (~16100 x g) for 5 min.
9. Transfer 120 µL of the supernatant to 1.5 mL tube. Take care not to take in any chloroform from the bottom- if this happens, put back everything in the original tube and centrifuge again.
10. Add 80 µL of cold Isopropanol to each tube. (Isopropanol should be stored at −20°C).
11. Precipitate at −20°C for 1 hr if to be continued on the same day or at 4°C overnight if to be continued on the next day.
12. Centrifuge at 4°C and 14000 rpm for 20 min.
13. Discard the supernatant; and re-suspend the pellet in 200 µL of 70% ethanol.
14. Vortex slightly/mix by hand for few seconds by inverting.
15. Centrifuge at 20°C and 14000 rpm for 5 min.
16. Discard the supernatant.
17. Re-suspend the pellet in 200 µL 95% ethanol.
18. Vortex slightly or hand mix by inverting.
19. Centrifuge at 20°C and 14000 rpm for 5 min.
20. Discard the supernatant. Carefully siphon off the remaining supernatant with a smaller calibrated pipette (20–200 µL). Discard tip for each sample.
21. Leave open the tubes for 30 min to 45 min on bench top.
22. Re-suspend the pellet in 30 µL of PCR water (or sterile distilled water) or in 1x TE buffer for long term storage.
23. Store DNA at −20°C.

DNA extraction from psyllids using REDExtract-N-Amp™ Tissue (or plant) PCR Kit from Sigma

The protocol using this kit is modified as below depending on the number of psyllids used per extraction.

1. Set water bath to 95°C before proceeding
2. Place psyllids in 1.5 mL tube as described in the CTAB method.
3. For extraction from single adult psyllid, add 10 µL of the extraction buffer and crush the psyllid with blunt 20–200 µL pipette tip (flame the tip and press against the cap of the sample tube to make it blunt).
4. Incubate at 95°C for 10 min.
5. Remove from the water bath and add 10 µL dilution buffer.
6. Store the extract at −20°C till further use.

7. For extractions from 2–3 adults add 15 µL of each of extraction and dilution buffer.
8. For extraction from five adults, add 25 µL of each buffer.
9. For nymphs, use 10 µL and 15 µL of each buffer for 2–3 and five nymphs respectively.

Conventional PCR (cPCR)

Primers OI1/OA1/OI2C (Table 1 and 2) detect '*Candidatus* Liberibacter asiaticus' and '*Candidatus* Liberibacter africanus' and gives an amplification band of 1160 bp. Distinction between the two species requires digestion of the amplicon with restriction endonuclease *X*baI. ''*Candidatus* Liberibacter asiaticus' produces two bands, 640 bp and 520 bp while '*Candidatus* Liberibacter africanus' produces three bands (520 bp, 506 bp, 130 bp).

Primers A2/J5 detects beta-operon region of '*Candidatus* Liberibacter africanus' and '*Candidatus* Liberibacter asiaticus'. Band size for '*Candidatus* Liberibacter africanus' is 669 bp and for *'Candidatus* Liberibacter asiaticus is 703 bp (Table 1 and Table 3).

Primers GB1 and GB3 detects 16S rDNA of '*Candidatus* Liberibacter americanus' but not that of '*Candidatus* Liberibacter asiaticus or '*Candidatus* Liberibacter africanus' (Table 1 and Table 4).

Duplex PCR using primers GB1/GB3 and A2/J5 is used for detection of '*Candidatus* Liberibacter africanus', '*Candidatus* Liberibacter americanus' and '*Candidatus* Liberibacter asiaticus' (Table 1 and Table 5).

Table 1. Primers for conventional cPCR to detect HLB pathogen, '*Candidatus* Liberibacter sp.'

Primer name	**Primer sequence (5' to 3')**	**Target gene**	**Reference**
Forward: OI1	5'GCG CGT ATG CAA TAC GAG CGG CA 3'	16S rDNA	Jagoueix et al. 1996)
Forward: OA1	5′-GCG CGT ATT TTA TAC GAG CGG CA-3′		
Reverse: OI2C	5' GCC TCG CGA CTT CGC AAC CCA T 3'		
Forward: A2	5′- TAT AAA GGT TGA CCT TTC GAG TTT -3′	rplA/rplJ	Hocquellet et al. 1999
Reverse: J5	5′- ACA AAA GCA GAA ATA GCA CGA ACA A -3′		
Forward: GB1	5′-AAG TCG AGC GAG TAC GCA AGT ACT-3′	16S rDNA	Teixeira et al. (2005)
Reverse: GB3	5′-CCA ACT TAA TGA TGG CAA ATA TAG-3′		

Table 2. cPCR reaction mix preparation for OI1/OIA/OI2C.

Reagents	µL/reaction	Final concentration	PCR condition
PCR water	12.8 µL		Initial denaturation at 94°C for 2 min; 35 cycles of: Denaturation at 92°C for 60 sec; Annealing & extension at 72°C for 90 sec; Final extension at 72°C for 10 min; Hold at 10°C
10x PCR mix (without MgCl2)	2.5 µL	1x	
MgCl2 (25mM)	2.5 µL	2.5 mM	
dNTP Mix (10mM each)	0.6 µL	0.24 mM each	
OI1 (10µM)	1.0 µL	0.4 µM	
OIA (10µM)	1.0 µL	0.4 µM	
OI2C (10µM)	1.0 µL	0.4 µM	
Taq (DNA polymerase (5µ/µl)	0.125	0.025U/µL	
DNA template	2 µL		
Total reaction volume	25 µL		

Table 3. PCR mix preparation for A2/J5

Reagents	µL/reaction	Final concentration
PCR water	13.8 µL	
10x PCR mix (without MgCl2)	2.5 µL	1x
MgCl2 (25mM)	2.5 µL	2.5 mM
dNTP Mix (10mM each)	0.6 µL	0.24 mM each
A2 (10µM)	1.0 µL	0.4 µM
J5 (10µM)	1.0 µL	0.4 µM
Taq (DNA polymerase (5µ/µl)	0.125	0.025U/ µL
DNA template	2 µL	
Total reaction volume	25 µL	

PCR conditions for A2/J5

Initial denaturation at 94°C for 2 min; 35 cycles each of: Denaturation at 92°C for 20 sec; Annealing at 62°C for 20 sec; Elongation at 72°C for 45 sec followed by a final extension at 72°C for 10 min (one cycle only) and hold at 10°C.

Table 4. PCR reaction mix for GB1 and GB3.

Reagents	µL/reaction	Final concentration
PCR water	13.8 µL	
10x PCR mix (without MgCl2)	2.5 µL	1x
MgCl2 (25mM)	2.5 µL	2.5 mM
dNTP Mix (10mM each)	0.6 µL	0.24 mM each
GB1 (10µM)	1.0 µL	0.4 µM
GB3 (10µM)	1.0 µL	0.4 µM
Taq (DNA polymerase (5 U/µl)	0.125	0.025 U/µL
DNA template	2 µL	
Total reaction volume	25 µL	

Table 5. Duplex of A2/J5 and GB1/GB3 (EPPO, 2021).

Reagents	µL/reaction	Final concentration
PCR water	10.375 µL	
10x PCR mix (without MgCl2)	2.5 µL	1x
MgCl2 (25 mM)	2 µL	2 mM
dNTP Mix (10 mM each)	0.5 µL	0.2 mM each
GB1 (20 µM)	1.25 µL	1µM
GB3 (20 µM)	1.25 µL	1µM
A2 (20 µM)	1.25 µL	1µM
J5 (20 µM)	1.25 µL	1µM
Taq (DNA polymerase (5U/µl)	0.125	0.625 U or 0.025 U/ µL
DNA template	2 µL	
Total reaction volume	25 µL	

PCR condition for duplex PCR of GB1/GB3 and A2/J5 (EPPO, 2021)

Initial denaturation at 96°C for 5 min followed by 35 cycles of: denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec and 72°C for 10 min; Final extension at 72°C for 10 min and cool/hold at 4°C.

Control for cPCR

- No Template Control (NTC) is a negative control that uses PCR water (no DNA template) with the PCR mix.
- Extraction control (EC) is a negative control consisting of plant/ insect known to be free of the liberibacters.
- Positive control (PC) is a known positive DNA of the specific species.

Result interpretation of cPCR

- A test result is positive if expected band size for the specific species are obtained:
- 1160 bp for CLaf or CLas using OI1/OIA/OI2C;
- 669 bp for CLaf and 703 bp for CLas using A2/J5;
- 027 bp for CLam, 669 bp for CLaf, 703 bp for CLas in the duplex PCR using GB1/GB3 and A2/J5.
- All negative controls do not produce bands
- Positive control shows the expected band size for the specific species: 1160 bp for CLaf or CLas using OI1/OIA/OI2C; 669 bp for CLaf and 703 bp for CLas using A2/J5; and 1027 bp for CLam, 669 bp for CLaf, 703 bp for CLas in the duplex PCR using GB1/GB3 and A2/J5.
- If any of the controls deviate from the above, then the tests should be repeated. Re-sampling may be required under certain circumstances.

Real-time PCR

Real-time PCR uses primers and probes developed by Li et al. (2006) for plant samples and Manjunath et al. 2008 for *D. citri* samples. An alternate primer for *D. citri* wingless gene developed by Li et al. 2008 can also be used (Table 6). A reverse primer and a probe common to all the three species are used. Internal control is provided by the plant DNA which is detected by the primer and probe for cytochrome oxidase (COX) gene. For psyllids, internal controls target the *wingless* gene of psyllids.

Table 6. Primers and probes based on 16S rDNA for detection of the three '*Candidatus* Liberibacter' species (Li et al., 2006) in plant and psyllid samples.

Primer/Probe name	**Primer sequence (5' to 3')**	**Reference**
Forward: HLBaf	5′-CGA GCG CGT ATT TTA TAC GAG CG-3′	Li et al., 2006
Forward: HLBam	5′-GAG CGA GTA CGC AAG TAC TAG-3′	
Forward: HLBas	5′-TCG AGC GCG TAT GCG AAT ACG-3′	Duan et al., 2009
Forward: CLas-4G	5′-AGT CGA GCG CGT ATG CGA AT-3′	Bao et al., 2020
Reverse primer: HLBr	5′-GCG TTA TCC CGT AGA AAA AGG TAG-3′	Li et al., 2006
probe: HLBp	5′-FAM-AGA CGG GTG AGT AAC GCG-BHQ1-3′	Li et al., 2006
Forward primer: COXf	5′-GTA TGC CAC GTC GCA TTC CAG A-3′	
Reverse: COXr	5′-GCC AAA ACT GCT AAG GGC ATT C-3′	
Probe: COXp	5′-TET-CAG ATG CTT ACG CTG-BHQ1-3′	
For *Diaphorina citri* use the following primer and probe instead of COX		
Forward primer : DCF	5′-TGG TGT AGA TGG TTG TGA TCT GAT GTG-3′	Manjunath et al., 2008
Reverse primer: DCR	: 5′-ACC GTT CCA CGA CGG TGA-3′	
Probe: DCP	5′-HEX-TGT GGG CGA GGC TAC AGA AC-BHQ1-3′	
For *Cacopsylla heterogena* use the following primer and probe instead of the primer and probe for *D. citri*.		
Forward primer: WGCaF	5'-GCA CTC AAG GAC CGG TTT GAC GG -3'	Om, 2017
Reverse primer: WGCaR	5'-ACT GCC GCG CAC ATT CCC CTC -3'	
Prober: WGp	5'-TET - TTA CTG ACC ATG ACT CTG GAC GC BHQ2-3'	Li et al., 2008

Preparation of PCR reaction mix

PCR reaction mix using the above primers and probes are given in Table 7.

Table 7. PCR reaction mix for detection of all three '*Canditatus* Liberibacter' species.

Reagents	**µL/reaction**	**Final concentration**	**Remarks**
PCR water	1.5 µL		Leave out forward primers of HLBaf and HLBam if routine work/detection for CLas is done; Replace HLBas with CLas-4G forward primer in case low titre of bacterium is suspected.
10x PCR mix (without MgCl2)	1.3 µL	1x	
MgCl2 (50 mM)	1.5 µL	6 mM	
dNTP Mix (10 mM each)	0.3 µL	0.24 mM each	
Primer Mix of HLBaf, HLBam, HLBas, HLBr (2 µM)*	1.5 µL	240 nM	
HLBp (1 µM)	1.9 µL	150 nM	
Primer Mix of COXf/COXr or DCF/DCR or WGCaF/WGCaR (2 µM)*	1.5 µL	240 nM	
COXp or DCP or WgP (1 µM)	1.9 µL	150 nM	
Taq (DNA polymerase (5 U/µl)	0.125 µL	0.025 U/µL	
DNA template	1 µL		
Total reaction volume	µL		

*see primer dilution

Real-time PCR conditions

Pre-incubation at 50^{o}C for 2 min followed by initial denaturation at 95^{o}C for 10 min and 40 cycles of: denaturation at 95^{o}C for 30 sec, followed by annealing and extension at 58^{o}C for 40 sec.

Real-time PCR result interpretation

- Test is valid only if:
- The positive control (PC) shows exponential amplification curve.
- Non template control (NTC) does not show an amplification curve or amplification curve is below the defined Ct value.
- The internal control e.g., COX/DC/WG should show amplification curves for COX or DC or WG depending on the sample type (COX for plant, DC for *D. citri*, WG for *C. heterogena*).
- A sample is positive if the sample shows an exponential amplification curve or has an amplification curve above the Ct value, provided all of the above conditions are met.
- If any of the above conditions are not met, the test should be repeated.
- If a sample does not show an amplification curve for HLB it could mean, provided all above conditions are met, that the DNA of the pathogen is not present in that sample and the results is noted as 'Not detected' instead of saying negative.

Sequencing should be performed using A2/J5 or any other cPCR primers to confirm the identification of bacteria species if samples are tested for detection of CLaf and CLam as these are

not detected in Bhutan till date. Include all three species if other species are being tested for acquisition and/or transmission of the CLaf, CLam and CLas.

References

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SoP-2: Diagnostic Protocol for Rice blast

Pyricularia oryzae Cavara 1892
Disease
Rice blast

Hosts
Hosts of P. oryzae include species of cereals (rice - Oryza sativa, African rice - Oryza glaberrima, perennial wild rice - Oryza longistaminata, finger millet - Eleusine coracana, fox tail millet - Setaria italic, fonio millet - Digitaria exilis, barley - Hordeum vulgare, Italian rye grass - Lolium multiflorum, common millet - Panicum miliaceum, wheat - Triticum aestivum, maize - Zea mays) and grasses (armgrass millet - Brachiara distachya, crabgrass - Digitaria sanguinalis, crowfoot grass or goosegrass - Eleusine indica, barnyard grass - Echinochloa crus-galli, cutgrass - Leersia hexandra, torpedo grass - Panicum repens, itchgrass - Rottboelia exaltata etc.

Taxonomic information
Name: *Pyricularia oryzae Cavara* 1892 (anamorph or asexual stage; teleomorph or sexual stage: *Magnaporthe oryzae* Couch (2002).

Taxonomic position:
Domain – Eukaryota
Kingdom – Fungi
Phylum – Ascomycota
Class –Sordariomycetes
Order – Magnaporthales
Family – Pyriculariaceae
Genus – Pyricularia
Species – Pyricularia oryzae (EPPO 2023).

Detection
Symptom
Pyricularia oryzae infects almost all the parts of rice plant: leaf (leaf blast), leaf collar (collar blast), culm, culm nodes (node blast), panicle nodes (neck blast or neck rot), and panicle (panicle blast). The pathogen can infect rice plant at any growth stage. Infection appears as lesion or spots.
Leaf blast: Initial symptom on leaf appears as white to grey-green lesions with green borders. Lesions become elliptical or diamond shaped with whitish to grey centres and brown to necrotic borders as they become old (Figure 1). Lesions can coalesce and become large and may kill the entire leaf.
Node infection: Dry dark-brown discolouration appears at the node of blast infected culm (Figure 1). The culm breaks off from the node killing the entire rice plant. When infection of node occurs at the junction of leaf blade and leaf sheath it is called collar rot, and when infection at the base of the panicle (the junction of stem and the seed head) it called neck blast. Infection on the neck causes unfilled grain which appears as white head a condition known as blanking (Figure 1).

Panicle blast: Gray-brown necrotic lesion appears on the panicles which also infects, spikes, spikelet, and panicle branches. The panicle appears as brown or dark head. As the infection matures, the panicles branches break at the lesion.

Confusion with other diseases and disorders

Brown spot

Initial symptoms of rice blast on leaves can be easily confused with brown spot symptoms. However, the basic difference is that the leaf blast lesions are spindle shaped with pointed ends whereas the brown spot lesions are round to oval in shape, brown in colour with yellow halo around the lesion (Figure 2). Brown spot is caused by *Bipolaris oryzae* (Breda de Haan) Shoemaker (teleomorph- *Cochliobolus miyabeanus*). Conidia of *B. oryzae* are 63-153 x 14-22 µm in size, curved with numerous septa (Figure 2).

Figure 1. Rice blast: (left) spindle shaped lesion on leaves; (centre) node blast; (right) neck blast with white head/panicle (photo: N.Om).

Rice stem borer

Rice stem borer infest rice plants at any growth stage. Larvae of stem borer burrow inside the culm and causes deadheart during vegetative stage and whitehead during reproductive stage. Whitehead symptom produced by neck rot can be easily confused with rice stem borer infestation. However, whiteheads caused by stem borer can be easily pulled off from the plant whereas whiteheads due blast cannot. Moreover, signs of stem borer such as pupae, holes and frass may also be also visible.

Figure 2. Brown spot: (left) oval shaped brown spots with yellow or necrotic borders; (right) conidia of *Bipolaris* sp. (Photo: N. Om).

Identification

Identification can be done both through morphological and molecular methods.

Morphological methods

Inducing sporulation on plant tissue

- Place fresh sample showing initial symptom of blast in a moist chamber (plastic bag or petri plate lined with wet tissue or blotting paper).
- Incubate for 28°C for 24 - 48 hrs. Incubate under light if sporulation fails. Try incubation on artificial media.
- Examine for sporulation: take a mass of mycelium and place on a glass slide loaded with water or cotton blue; observe under 10x or higher for the typical pyriform conidia of *P. oryzae*.

Inducing sporulation on media

Sporulation *P. oryzae* can be isolated on water agar or Potato Dextrose Agar (PDA) which can be sub-cultured on PDA. Other media for sporulation of *P. oryzae* includes cornmeal-rice straw agar, oatmeal agar and cornmeal agar.

Morphological characteristics

Conidia (spores) solitary, pyriform (pear-shaped) to obclavate with 2 septa, 25-33.5 x 7-11 µm, each conidium bears a basal appendage called hilum which is 0.5–1 x 1–2µm. Conidiophores septated and brown (Figure 3). Variation in conidia shapes have been reported ranging from oval, short pyriform, pyriform and long pyriform (Figure 4).

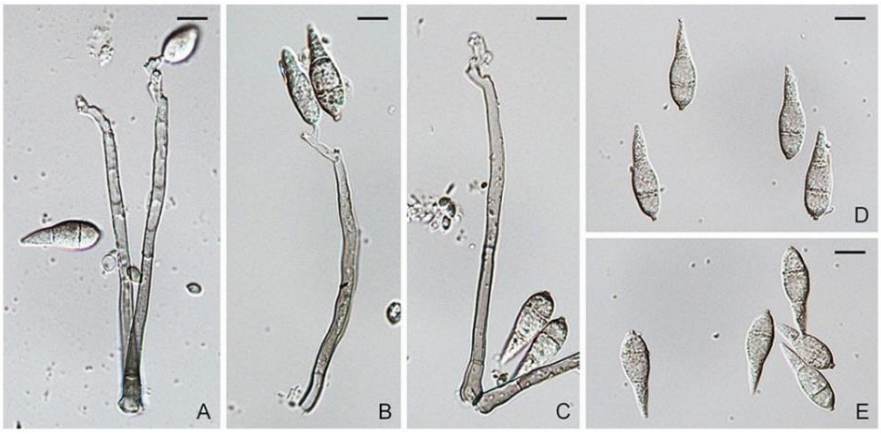

Figure 3. Conidiophores and conidia of *P. oryzae* (A-E) (Luo and Zhang, 2022)

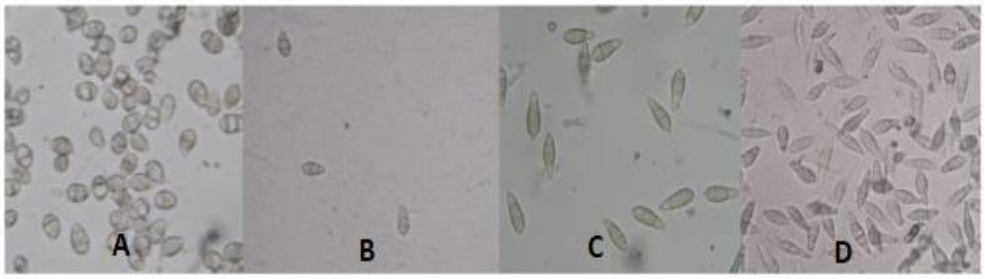

Figure 4. Variation in conidia shapes of *P. oryzae*: (A) oval; (B) short pyriform; (C) pyriform; (D) long pyriform (Longya et al., 2020).

Molecular methods

Laboratory requirements

- 2.0, 20, 200, and 1000 µL sterile pipette tips (prefer barrier tips)
- Microcentrifuge
- Microcentrifuge tubes 1.5 and 2.0 mL.
- Benchtop vortexer.
- 0.2 mL PCR tubes
- Thermocycler
- Gel tray with suitable comb/s, electrophoresis tank and powerpack
- UV transilluminator; Camera/gel documentation system

- PCR reagents
- Running buffer, 0.5 - 1x TBE (see NPPC protocol on buffer preparation)
- Agarose
- DNA ladder
- DNA staining agent (e.g., GelRed, avoid use of ethidium bromide) & Gel loading dye

DNA extraction

Refer specific DNA extraction methods provided by the manufacturer if using a commercial extraction kit (e.g., Bioline, Puregene, Qiagen, Sigma, etc.). Refer NPPC protocol on DNA extraction from fungi and fungi-like organism method.

Prepare PCR mix reaction as per the protocol received with the DNA Taq DNA polymerase or use the protocol in Table 1.

Use the DNA to amplify actin, beta-tubulin and calmodulin gene regions using the primer pairs and PCR conditions in Table 1. Run the PCR product in a TBE gel. After the gel electrophoresis, PCR product should produce a band at 320bp, 550bp and 500bp for actin, beta-tubulin and calmodulin gene region respectively. The PCR products should be purified using PCR Purification Kit by following manufacturer's protocol. The purified products should submit to DNA sequencing facilities (Macrogen Inc. or others). The DNA sequence data is BLAST analyzed in NCBI and compared with reference sequence data listed in Table 3 for confirmation.

Table 1. PCR mix preparation

Reagents	**µl/reaction**	**Final concentration**
PCR water	17.3	
10x PCR mix (without MgCl2)	2.5µl	1x (2mm MgCl2)
MgCl2 (25mM)	0.5	
dNTP Mix (10mM each)	0.6 µl	0.24mMeach
Primer 1 (10µM)	1µl	0.4µM
Primer 2 (10µM)	1µl	0.4µM
Taq (DNA polymerase (5µ/µl)	0.125µl	0.025U/µl
DNA template	2 µl	
Total reaction volume	25 µl	

Table 2: Primer pair for amplification of different gene region (Couch and Kohn, 2002).

Locus	Amplicon	Primer pair	Primer Sequence
Actin	320bp	ACT-512F	5'-ATGTGCAAGGCCGGTTTCGC-3'
		ACT-783R	5'-TACGAGTGCCTTCTGGCCCAT-3'
Beta-tubulin	550bp	Bt1a	5'-TTCCCCCGTCTCCACTTCTTCATG-3'
		Bt1b	5'-GACGAGATCGTTCATGTTGAACTC-3'
Calmodulin	500bp	CAL-228F	5'-GAGTTCAAGGAGGCCTTCTCCC-3'
		CAL-737R	5'-CATCTTTCTGGCCATCATGG-3'
PCR conditions: Initial denaturation: 1 cycle at 98ºC for 30 sec; 30 cycles of: denaturtion at 98ºC for 10 sec; annealing at 60ºC for 30 sec; extension at 72ºC for 30 sec; Final extension at72ºC for 5 min; Hold at 10ºC			

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SoP-3: Diagnostic Protocol for Chilli blight

Phytophthora capsici Leonian (1992)

Disease

Common name is *Phytophthora* blight. In Bhutan, it is referred to as chilli blight.

Hosts

Though primarily thought to affecting chilli, *Phytophthora capsici* has a wide host range affecting other plants in Solanaceae, and Cucurbitaceae and Fabaceae (Lamour et al. 2012). It is widely distributed (Erwin and Ribeiro, 1996; Sun et al, 2008).

Taxonomic information

Name: *Phytophthora capsici* Leonian (1922)

Taxonomic position:

Domain – Eukaryota
Kingdom – Chromista
Phylum – Oomycota
Class –Oomycetes
Order – Peronosporales
Family – Peronosporaceae
Genus – Phytophthora
Species – Phytophthora capsici

Detection

Detection of the pathogen can be based on symptoms, morphological and molecular characters.

Symptoms

The pathogen can infect all parts of the plant. Infection can occur in the nursery as well as in transplanted fields. Infected parts appear as dark brown lesions on stems, branches, crown, and roots (Figure 1). Infection results in rotting of tissues and leads to wilting of plants (Figure 1). Infection in the nurseries results in damping-off, leaving patches of dead seedlings. Infected plant parts exhibit water-soaked and dark brown lesions. On leaves, symptoms start as light green and turns yellowish as disease advances. Leaf spots may start from the leaf margin that is in contact with the soil or may also occur due to spores splashed from the soil or nearby infected tissues. On fruits or chilli pods, water-soaked lesions appear and results in soft fruits. White powdery growth may form on infected fruits.

Morphology

There are number of morphological characters used for identification of *P. capsici*. Like all other species of *Phytopthora*, the morphological characters used for *P. capsici* include sporangium shape, size, length:width ratio, papillation, caducity; shape of the sporangia Infected tissues under

warm and wet condition produce lemon-shaped sporangia. There are number of morphological characters used for identification of *P. capsici*. Like all other species of *Phytopthora*, the morphological characters used for *P. capsici* include sporangium shape, size, length:width ratio, papillation, caducity; shape of the sporangia Infected tissues under warm and wet condition produce lemon-shaped sporangia. On culture media, sporangia production must be induced by incubating cultures submerged in sterile distilled water exposed to light.

P. capsici is most commonly found in asexual stage. Asexual reproduction produces sporangia which are microscopic sac-like structures that house spores known as zoospores. Zoospores are flagellated and therefore are motile in the presence of free moisture. Zoospore release from the sporangia can be induced (in the laboratory) by incubating cultures at low temperature e.g., 4°C for 10-15 min.

Sexual spores or oospores can also be observed if mating type A1 and A2 are present (on infected tissues or under laboratory conditions) and have contacted each other. Oospores are sexual resting spores which can persist in soil for many years and can germinate forming mycelia and also sporangia under wet conditions.

Figure 1. Symptoms of *Phytophthora* blight: dark brown lesions on stem, crown and root, and wilting of plants.

Isolation

Phytophthora capsici can be isolated from soil, irrigation water, and plant tissues. Isolation from infected plant tissue is the easiest as isolation from soil and water requires baiting the pathogens from these sources using baits such as rhododendron leaves and susceptible plants like egg plants, tomatoes and chilli pods.

Isolation and establishment of pure culture of *P. capsici* require selective media. Selective media such as V8, CA and MEA media are used. At the NPPC, *P. capsici* from plant tissue has been isolated using carrot agar (CA). Isolation can be initiated with direct culture on media or transfer from moist chamber incubation. Moist chamber incubation is useful for initiating sporulation.

Selective carrot agar (CA) media for isolation of *Phytophthora capsici* from plant tissue

- Grind 200g of washed carrot slices in warm water, just enough to cover the carrot slices, using a kitchen blender.
- Filter the ground carrot through four layers of cheese cloth to obtain the carrot juice.
- Place 15g of agar in a 2L flask
- Add the carrot juice to the flask with agar. Adjust the final volume of liquid to 1L with distilled or RO water and mixed well.
- Autoclave at 121°C for 15 min.
- Cool the autoclaved media to 50-60°C.
- Add antibiotics to suppress growth of bacteria and other fungi to the cooled media.
- Then pour into sterile petri plates inside a laminar flow. Let the plates dry in the laminar flow for ~30 min. Wrap the plates using plastic bags or cling wrap and store in the fridge till further use.
- Alternately, autoclaved media can be stored on the shelves in the flask for pouring later.

Note: media amended with antibiotics (or fungicides) must be used within 2-3 weeks.

- In NPPC, nystatin is the only antibiotic (antifungal) used:
- Prepare antibiotic stock as by dissolving 500mg (powder or tablet) in 5ml of sterile distilled/RO water that give a stock concentration of 100mg/ml. Then add 0.25ml of the stock solution to 1L media to achieve a final concentration of 25 µg/ml in 1L media (Drenth and Sendall, 2001).

Direct incubation in moist chamber

- Wash the plant tissue (e.g., leaves or fruits or roots or whole plant if seedlings) in running water. Rinse with sterile distilled water.
- Flame sterilises all tools e.g., forceps, loops, needles.
- Cut ~5 – 10 cm length of the plant tissue, or whole leaf for leaf samples, at the edge of diseased part including a few cm of the tissue.
- Surface sterilise tissue with 1% bleach (sodium hypochlorite) or 70% ethanol by dipping the tissue for 30-60 secs. Whole tissue must be covered in ethanol. Rinse away ethanol with sterile distilled water 2-3 times. Drain off excess water from the samples and blot dry using sterile paper towel.
- Place plant tissue in a petri dish lined with wet paper towel/filter paper or filled with shallow distilled water.
- Incubate under light at 18-25°C for 24-48 hrs.
- Examine for sporangia production and if present, then transfer the infected part onto a selective media.

Note: sometimes, it is desirable to skip step 4 and incubate plant tissues directly after washing.

Plating infected plant tissues on selective media

- Wash soil and other dirt off the sample in running water.
- Cut small section of the plant sample (whether leaf or stem or root), of around 0.5-1cm^2 in size, from the edge of the diseased area. Sample should include both diseased and the

adjacent healthy tissue. If it is a stem or root sample of >1 cm diameter, it may be desirable to use the outer layer or bark only.

- Sterilise all tools and plant material as above (Section B).
- Blot dry plant tissues after rinsing.
- Transfer to selective media CA. Usually four pieces of 0.5-1cm^2 samples (from step 2) are plated onto each plate at equidistance. Press the tissue into the medium to ensure good contact between the tissue and the antibiotics in the medium that will supress bacterial growth.
- Incubate at 28°C in the dark (use an incubator) and observe every day for growth of non-septate hyphae. If other fungi are observed to grow, then transfer plugs of agar from active region, making sure that the plug contains only one type of growth, to another plate with selective media. Once a pure culture is obtained. Observe for hyaline non-septate hyphae.

Inducing sporangia formation in plates

- From the pure culture of an isolate which is < 7 days old, transfer single agar plug to petri plates with basal media of CA or CMA or V8 juice. Incubate at 28°C. Examine cultures daily.
- When growth is about half of the petri plate, transfer 6-10 agar plugs into sterile empty petri plate.
- Flood the petri plate with sterile distilled water covering the surface of the agar plugs.
- Incubate at room temperature (~20-25°C) under continuous light. Examine after 12-24 hrs for mycelial growth and sporangia formation under a dissecting microscope.
- If sporangia are not formed after 24-48 hrs. It is probably not *Phytophthora*.
- If difficult to see under a dissecting scope, take a small mycelial growth on a glass slide and observe under higher magnification using a compound microscope. Sporangia of *Phytophthora* are lemon-shaped. Sporangia of *Pythium* are globose and more irregular.
- Once sporangia formation is confirmed, proceed for characterisation and identification of the isolate.

Identification of isolates

Once an isolate is established to be that of *Phytophthora*, proceed to identification of the species based on morphology and molecular characteristics. Morphological characters include:

Colony morphology on standard media e.g., PDA.

Sporangial morphology:

- Sporangiophore characters
- Hyphal swelling
- Oospores
- Lack of chlamydospores

Note: Culture for identification and subsequent work such as pathogenicity and inoculum production must preferably be obtained from single hyphal tip, or single spore. Culture obtained in such manner should be incubated at the optimum temperature (~28°C) on basal medium such CA or CMA or V8 juice but not PDA. Sporangia production and other work should follow.

Colony morphology

- Whether mycelium is on the surface (appressed) or aerial, submerged.
- Coenocytic hyphae, branch at nearly right angles and sometimes constricted at the base.
- Hyphal swelling.
- Chlamydospores are not common in *P. capsici*.

Sporangial morphology (Drenth and Sendall, 2001; Abad et al., 2023)

- Sporangia shapes are ovoid, obivoid, ellipsoid (Figure 2).
- Dimension of sporangium (length to breadth ratio). Length and breadth of at least 25 sporangia must be measured to confirm the shape.
- Sporangiophore bears the sporangia. Sporangiophores are unbranched or irregularly branched occasionally simple sympodia (Figure 3).
- Hyphal swellings occasionally produced in aqueous cultures; Lacks chlamydospores
- Presence of an apical thickening of the sporangium called papilla (nipple). *P. capsici* has sporangia that are papillated or semi- papillated, sometimes with 2-3 apices (Figure 3).
- Presence or absence of caducity (shedding of sporangium at maturity). Crush the agar or mycelial growth taken from agar in Section C step 7 to dislodge sporangia and observe if the sporangia get detached from sporangiophores.
- Length of the pedicel on the shed sporangia: short (< 5um), intermediate (5-10um) or long (> 10um) (Figures 3). The pedicel (sporangial stalk) is the hyphal strand left behind on the sporangium when it is detached from the sporangiophore. Pedicels of at least 25 sporangia must be measured to get the average length.

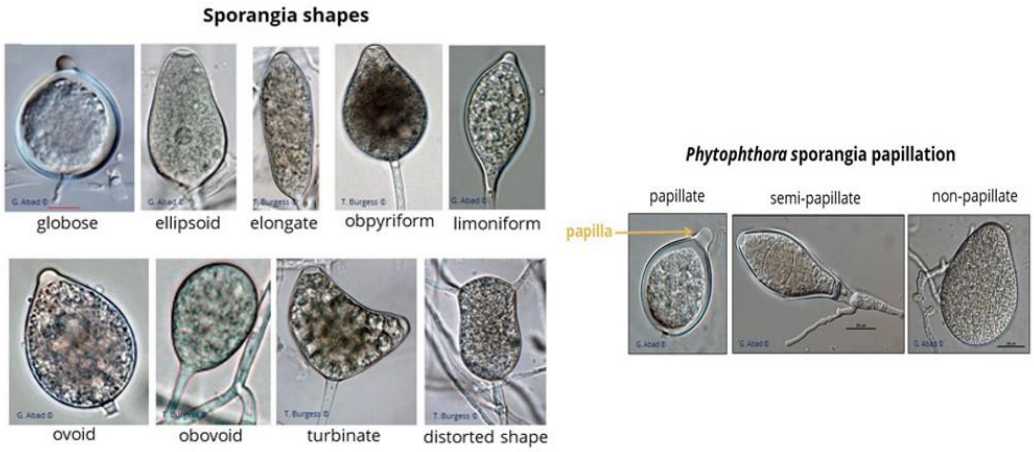

Figure 2. Shape and papillation of sporangia of *Phytophthora* (images from Abad et al. 2023).

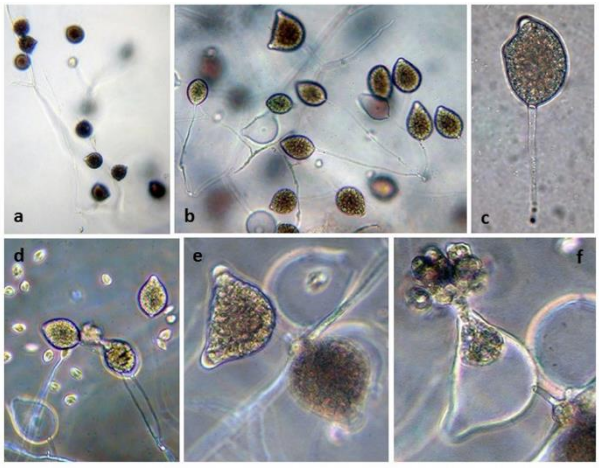

Figure 3. *Phytophthora capsici*: (a) sporangia originated in unbranched and simple sympodial sporangiophores, (b) sporangia originated in unbranched and irregular branched sporangiophores, (c) semipaillate ellipsoid caducous sporangium with long pedicel, (d-f) zoospores produced from semipapillated or bipapillate sporangia (images and description from Abad et al. 2023).

Morphology of sexual spores

- Male structure: antheridium
- Female structure: Oogonia
- Spores formed as a result of fusion of male and female are called as oospores.
- Requires mating types A and B to confirm the mating type of the isolate as well as to produce oospores. Paring of the target isolate with a known mating type will help determine the mating type of the isolate.
- Oospores production will take long, around 30 days.

Staining of sporangia/oospores/any structure

For observation under higher magnification using a compound microscope, take pieces of agar plug with mycelia and smashed onto a glass slide, stain with lacto-phenol blue OR fix and stain sporangia as below:

- Prepare a solution of 0.05-0.1% of acid fushsin in 85% lactic acid (full strength).
- Immerse agar plugs (containing mycelia/sporangia/sexual structures) in this solution overnight.
- Mount mycelium mat in clear lactoglycerol for microscopic examination
- Measure at least 25 structures of each type (whether sporangia for size & shape or pedicel length OR oospores).

Molecular

Molecular identification is done using ITS rDNA and COI genes from DNA of culture samples. Primers and PCR mix preparation for conventional PCR are given in Table 1 and 2 respectively.

Table 1. Primers for conventional PCR for detection of *P. capsici.*

Primer name	Primer sequence (5' to 3')	Target gene &	Reference
Forward: ITS5	5' – GAAGTAAAAGTCGTAACAAGG – 3'	ITS rDNA	White et al. 1990.
Reverse: ITS4	5' – TCCTCCGCTTATTGATATGC – 3'		
Forward: PC1	5'– GTCTTGTACCCTATCATGGCG – 3'	ITS rDNA (region 1)	Zhang et al. 2006
Reverse: PC2	5'– CGCCACAGCAGGAAAAGCATT– 3'		
Forward: COIF-1	5' – TCAWCWMGATGGCTTTTTTCAAC – 3'	Cytochrome oxidase I	Robideau et al 2011
Reverse: COIR-1	5' – RRHWACKTGACTDATRATACCAAA – 3'		

Table 2. cPCR mix preparation

Reagents	µL/reaction	Final concentration
PCR water	12.8 µL	
10x PCR mix (without MgCl2)	2.5 µL	1x
MgCl2 (25mM)	2.5 µL	2.5 mM
dNTP Mix (10mM each)	0.6 µL	0.24 mM each
Forward primer (10µM)	1.0 µL	0.4 µM
Reverse primer (10µM)	1.0 µL	0.4 µM
Taq (DNA polymerase (5µ/µl)	0.125	0.025U/µL
DNA template	2 µL	
Total reaction volume	25 µL	

PCR conditions (Ristaino et al. 1998, White et al. 1990, Zhang et al. 2006):

F**or ITS5/ITS4:** The thermal cycling parameters were initial denaturation at 96°C for 2 min followed by 35 cycles consisting of denaturation at 96°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. A final extension at 72°C for 10 min.

ITS5/IT4 gives a PCR product size of 899 bp.

For PC1/PC2: Initial denaturation of one cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 30 sec, 70°C for 30 sec and 72°C for 30 sec. A 7-min final extension at 72°C. Hold at 4-10°C.

PC1/PC2 gives a band of 560 bp.

Refer molecular identification of *Phytophthora* online at https://idtools.org/phytophthora/index.cfm?pageID=1877. The website provides information on morphology with images and detailed SoPs from isolation to culture storage, and molecular

methods and protocols required for conducting molecular identification including sequencing analysis using ITS and COI regions. The website has links for factsheet on over 100 species of *Phytophthora.*

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SoP-4: Diagnostic Protocol for Apple scab

Venturia inaequalis (Cooke) Winter

Disease

Apple scab

Hosts

Venturia inaequalis primarily infects cultivated apple varieties but can also infect other wild relatives of apple like crab apple, hawtorn (*Crataegus* Spp.*)*, firethorn (*Pyracantha* Spp.), mountain ash *(Sorbus* Spp.), *Cotoneaste*r spp, Vibutnum and loquat (*Eriobotrya japonica)* (CABI, 2021).

Taxonomic information

Domain – Eukaryota

Kingdom – Fungi

Phylum – Ascomycota

Class – Dothideomycetes

Order – Venturiales

Family – Venturiaceae

Genus – *Venturia*

Species – *Venturia inaequalis*

Distribution

Apple scab is present in many countries of Africa, Asia, Europe, North America, Oceania and South America.

Detection

Symptoms

Apple scab can affect leaves, petioles, sepals**,** blossom, fruits and pedicel.

Foliar symptoms (Figure 1): Lesions on leaves may occur singly or scattered over the blade. Lesions may coalesce cover and develop on either side of the leaf surface. Lesions first appear on the underside of the leaf as the first infection occurs at bud break. Lesions appear as light green compared to the healthy surface of the leaf. Lesions soon turn olive green and appear velvety. Some lesions turn black in colour and it may not have a definite margin. When observed under lens (10x), dark diffused mat-like fungal growths are visible.

Figure 1**.** Dark velvety lesions on leaf: (Leaf and middle) – lesion on upper side leaf surface; (right) – lesion on underside leaf surface.

Fruit symptoms: Apple fruit is most susceptible when young and infection on young apples result in development of the largest lesion. Fruit lesions are usually very small at first. Compared to leaf lesions, fruit lesions grow more slowly, darker coloured and more sharply bordered. Older lesions become bare, brown and corky in the centre. Cracking and deformation are common. Dropping may also happen when infected pedicel is girdled. When infection occurs late in the season, lesions are pin-head size and may not be visible till during storage.

Shoot and Twig symptoms: Pustules on young shoots appear as light brown blister like swellings similar in appearance to scab on bud scales, petioles and fruits in storage. Blisters develop that become olive-coloured as the fungus grows and ruptures the cuticle forming whitish ring around the pustules. The twigs and shoots infected also exhibit symptoms of cankers and dieback. Therefore, the trees become stunted and weakened reducing fruit production.

Figure 2. Scab lesions on fruits (Photos: N.Om & S. Chophel)

Identification

Confirmation of the disease is performed through examination of plant samples in the laboratory. Confirmation can be achieved through morphological and molecular techniques**.**

Morphological techniques

Collection of samples

- Collect infected samples e.g., leaf or fruits
- Place samples in paper bags with labels: Location name & GPS coordinates; Variety name; date of collection; collector's name.
- Wrap the paper bags in polythene and keep at 4°C till further use.

Materials required

- Leaf samples with visible symptoms of apple scab
- Microscope
- Scalpel (fine tip) or Razor blade
- Inoculating needles
- 70% ethanol
- Petri dishes
- Potato Dextrose Agar (PDA) or other suitable fungal growth medium
- Incubator

Visual inspection of samples

- Examine samples under the stereomicroscope and look for presence of dark mat of mycelium. If leaf samples, it is better to examine both upper and lower surfaces as mycelium growth on lower leaf surface is quite common. The mycelium mat contains numerous scab spores (conidia).
- Scrape of small section of the mycelium mat with help of scalpel, razor blade or inoculating needle.
- Place a small drop of cotton blue on a microscope slide and place the sample on the slide, cover with a cover slip and observe under a compound microscope
- Typical conidia of apple scab pathogen are single celled, uninucleate, and narrower at one end than other like a bowling pin (Figure 3).
- Mass of conidia appears dark or brown than single conidia. Size range between 6 and 12 µmin width, and 12 and 22 µmin length. Conidia are produced by special hyphae called conidiophores (Gauthier, 2018).

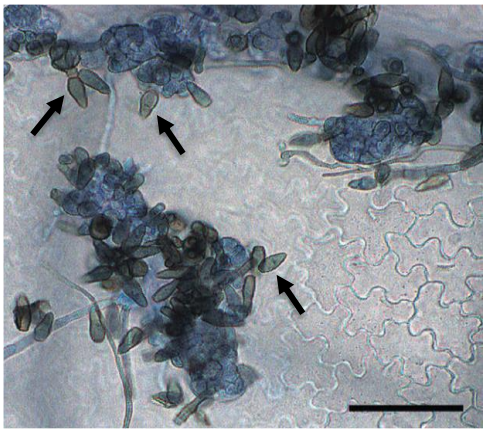

Figure 3. Conidia of *V. inaequalis* (Bowen 2011)

Isolation and sporulation

Moist chamber method: Sample can also be incubated in moist chamber to induce sporulation for direct examination of samples:

- Line a petri dish with blotting paper/filter paper (plastic bags or plastic containers can be substituted for petri dish especially if the samples are bulky e.g., fruits or tubers).
- Add a few drops of sterile distilled water using a dropper or a wash bottle to moisten the filter paper. Alternately, filter paper can be dipped in sterile water before placing in the petri dish; tip off excess water from the petri dish. Blotting /filter paper should be moist but not overflowing with water.
- Place two or more sterile wood tooth-picks on the moist filter paper.
- Place sample (e.g., leaf with lesions) on the tooth-picks.
- Incubate at 28°C for up to a week.
- Check for sporulation within every 24 hr.
- Typical conidia will be formed if the infection is due to apple scab pathogen.

Isolation on artificial media

- Flame scalpels, forceps and inoculating needles to sterilize before use.
- Cut small section of the plant sample (whether leaf or fruit), of around 0.5-1cm^2 in size, from the edge of the diseased area. Sample should include both diseased and the adjacent healthy tissue.
- Surface sterilise sample with 1% bleach (sodium hypochlorite) or 70% ethanol by dipping the tissue for 30-60 secs. Whole tissue must be covered in bleach or ethanol. Rinse two to three times with sterile distilled water. Drain off excess water from the samples and blot dry using sterile paper towel.
- Transfer to PDA. Usually four pieces of 0.5-1cm^2 samples (from step 2) are plated onto each plate at equidistance. Press the tissue into the medium to ensure good contact between the tissue and the antibiotics in the medium that will supress bacterial growth.

- Incubate at 28°C for up to 7 days. Subculture on PDA and incubate 28°C for 7-10 days.
- Apple scab caused by fungus *Venturia inaequalis* will appear as dark fuzzy colonies on the plate.
- Prepare slides and examine under microscope to check for conidia of *V. inaequalis*.
- For long term storage and for use in future, mix spores in 10% glycerol in preservation vials and store cultures at low temperature (−20 to −80°C).

Molecular method

Conventional polymerase chain reaction (PCR) and real-time PCR using TaqMan probes are described here for detection of *V. inaequalis*. Confirmation must be done through sequencing. Primers for cPCR are provided in Table 1. Use at least two out of the six primer sets. Reaction mix preparation is provided in Table 2.

Table 1. Primers for detection of *V. inaequalis* using cPCR. Same primer set used for sequencing unless stated.

Primer	Sequence	Target gene	Reference
Forward: ITS5	5' – GGAAGTAAAAGTCGTAACAAGG – 3'	Internal transcribed region	White et al. 1990; (use either ITS5 or ITS 1 as the forward primer with ITS4 as reverse).
Forward: ITS1	5′ – TCCG TAGGTGAACCTGCGG – 3′		
Reverse: ITS4	5' – TCCTCCGCTTATTGATATGC – 3'		
Forward: EFCF1	5' – AGTGCGGTGGTATCGACAAG – 3'	*Elongation factor alpha 1 (EF-1a)*	EPPO Bulletin 2016
Reverse: EFCF2	5' – TGCTCACGGGTCTGGCCAT – 3'		
Forward: ELF1	5' – CGAGAAGTTCGAGAAGGT – 3'		Prencipe et al. 2020
Reverse: ELF2	5' – CCAATGACGGTGACATAG – 3'		
Forward: TUB2Fd	5' – GTBCACCTYCARACCGGYCARTG – 3'	*Nuclear beta-tublin (TUB2)*	Groenewald et al. 2013
Reverse:TUB2Rd	5' – CCRGAYTGRCCRAARACRAAGTTGTC – 3'		
Forward: CAL-228F	5' – GAGTTCAAGGAGGCCTTCTCCC – 3'	*Nuclear calmodulin (CALM)*	Carbone & Kohn 1999
Reverse: CAL -737R	5' – CATCTTTCTGGCCATCATGG – 3'		
Forward: ACT-512F	5' – ATGTGCAAGGCCGGTTTCGC – 3'	*Nuclear actin ACT*	Carbone & Kohn 1999
Reverse:ACT-783	5' – TACGAGTCCTTCTGGCCCAT – 3'		

Fungal DNA extraction

Laboratory requirements

- 2.0, 20, 200, and 1000 µL sterile pipette tips (prefer barrier tips)
- Microcentrifuge
- Microcentrifuge tubes 1.5 and 2.0 mL.
- Benchtop vortexer.

- 0.2 mL PCR tubes
- Thermocycler
- Gel tray with suitable comb/s, electrophoresis tank and powerpack
- UV transilluminator; Camera/gel documentation system
- PCR reagents
- Running buffer, 0.5 - 1x TBE (see NPPC protocol on buffer preparation)
- Agarose
- DNA ladder
- DNA staining agent (e.g., GelRed, avoid use of ethidium bromide) & Gel loading dye

DNA extraction from plant tissue:
Refer NPPC protocol on DNA extraction from plant tissue.

DNA extraction from pure culture

- Culture sample on PDA for 12 – 15 days. Obtain pure culture by single spore culturing.
- Slice off ~2 cm^2 piece of agar of the pure culture.
- Place the mycelial piece in 2 mL tube (screw cap tubes are required if using FastPrep).
- Grind the sample using sterile micro pestle or any available grinding instrument e.g., using beating beads in FastPrep.
- Refer NPPC protocol on DNA extraction from fungi and fungi-like organism method for CTAB method of DNA extraction OR the manufacture's instruction for commercial kits.

PCR reaction mix preparation

Table 2. cPCR mix preparation

Reagents	**µL/reaction**	**Final concentration**
PCR water	13.8 µL	
10x PCR mix (without MgCl2)	2.5 µL	1x
MgCl2 (25mM)	2.5 µL	2.5 mM
dNTP Mix (10mM each)	0.6 µL	0.24 mM each
Forward primer (10µM)	1.0 µL	0.4 µM
Reverse primer (10µM)	1.0 µL	0.4 µM
Taq (DNA polymerase (5µ/µl)	0.125	0.025U/ µL
DNA template	2 µL	
Total reaction volume	25 µL	

PCR conditions
For ITS5/ITS4: Initial denaturation at 95 °C for 5 min, followed by 30 cycles of: denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec, extension at 72 °C for 1 min 40 sec, and one cycle of final extension at 72°C for 10 min. hold at 4-10°C. DNA band size (on gel and sequencing) is 550 – 1700 bp.

For ITS1/ITS4: Initial denaturation step at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing temperature of 54 °C for 1 min and a final extension step at 72 °C for 1 min. Hold at 4-10 °C.

For elongation factor alpha 1 (EF-1a):
EFCF1/EFCF2: Initial denaturation at 95 °C for 5 min, followed by 40 cycles of: denaturation at 94 °C for 30 sec, annealing at 52°C for 30 sec, extension at 72 °C for 30 sec, and one cycle of final extension at 72 °C for 10 min. Hold at 4-10 °C. DNA band size (on gel and sequencing) is about 680 bp. Same primer pair used for sequencing.

ELF1/ELF2: Initial denaturing step of 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 40 sec, 58 °C for 50 sec, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. Hold at Hold at 4-10 °C.

TEF1 is a protein coding region and thus standard code should be applied for translation.

For *TUB2* using primer TUB2Fd/TUB2Rd: Initial denaturation at 95 °C for 5 min, followed by 40 cycles of: denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec, extension at 72 °C for 30 sec, and one cycle of final extension at 72 °C for 10 min. Hold at 4-10 °C. DNA band size (on gel and sequencing) is about 450 bp. Same primer pair used for sequencing. TUB2 is a protein coding region and thus standard code should be applied for translation.

For *CALM* using primer CAL-228F/CAL737R: Initial denaturation at 95°C for 5 min, followed by 40 cycles of: denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec, extension at 72°C for 30 sec, and one cycle of final extension at 72°C for 10 min. Hold at 4-10°C. DNA band size (on gel and sequencing) is about 520 bp. Same primer pair used for sequencing. ***CALM*** is a protein coding region and thus standard code should be applied for translation.

For nuclear actin (ACT) gene using primer ACT-512F/ACT-783: Initial denaturation at 95°C for 5 min, followed by 40 cycles of: denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 30 sec, and one cycle of final extension at 72°C for 10 min. Hold at 4-10°C. DNA band size (on gel and sequencing) is about 290 bp. Same primer pair used for sequencing. ***ACT*** is a protein coding region and thus standard code should be applied for translation.

For COI gene using primer OomACT-512F/ACT-783: Initial denaturation at 95°C for 5 min, followed by 40 cycles of: denaturation at 94°C for 30 sec, annealing at 52°C for 30 sec, extension at 72°C for 30 sec, and one cycle of final extension at 72°C for 10 min. Hold at 4-10°C. DNA band size (on gel and sequencing) is about 290 bp. Same primer pair used for sequencing. ***ACT*** is a protein coding region and thus standard code should be applied for translation.

Primers and probes based on translation elongation factor1 alpha (*EF1-α*) for real-time PCR are provided in Table 3 and PCR reaction mix preparation in Table 4.

Fungal DNA (for real-time PCR)

DNA extraction method in the previous section can also be applied for extracting DNA for real-time PCR. Alternately, isolate on malt extract agar (MEA) and obtain pure culture on MEA and incubate for 30 days at 20 °C in the dark. Use at least 100 mg of fresh weight mycelium for DNA extraction follow the rest of the DNA extraction as in CTAB method or manufacturer's instruction.

Real-time PCR condition

Initial denaturation at 95 °C for 10 min followed by 40 cycles of 57 °C for 1 min and 95 °C for 15 sec.

Table 3. Primers and probes for detection of *V. inaequalis* from fungal spores (Prencipe et al. 2020).

Primer/Probe Name	Sequences (5' to 3')	Target gene	Reference
Forward primer: F1	5' – C ACTTCCCCGCTATTCACGT – 3'	Elongation factor 1	Prencipe et al. 2020
Reverse primer: R11	5' – GCAATCGTTAGCATCGTCATAGTG – 3'		
Probe: Ven1	[FAM]– CTCAAGGCAGCCCAACTTTCTCCGGT–[BHQ1]		

Table 4. Real-time PCR mix preparation

Reagents	µL/reaction	Final concentration
PCR water	14.78 µL	
10x PCR mix (without MgCl2)	2.5 µL	1x
MgCl2 (25mM)	2.5 µL	2.5 mM
dNTP Mix (10mM each)	0.6 µL	0.24 mM each
Forward primer ELF1 (3µM)	0.4 µL	3 µM
Reverse primer ELF2 (3µM)	0.4 µL	3 µM
Ven1 probe (5µM)	0.2 µL	
Taq (DNA polymerase (5µ/µl)	0.125	0.025U/ µL
DNA template	1 µL	
Total reaction volume	20 µL	

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SoP- 5: Diagnostic Protocol for Potato Bacterial wilt

Ralstonia solanacearum species complex (Smith) Yabuuchi, Kosako, Yano, Hotta & Nichiuchi

Disease

Bacterial wilt of potato or brown rot of potato; moko disease of banana and many more.

Hosts

Ralstonia solanacearum species complex has a wide host range that includes many cultivated and wild plants, and ornamentals and weed species. Important hosts are aubergine (*Solanacearum melongena*) banana and plantain (*Musa* spp.), and chili (*Capsicum annuum*), potato (*Solanacearum tuberosum*) and tomato (*S. lycospersicum*).

Taxonomic information

Name: *Ralstonia solanacearum* (Smith) Yabuuchi et al. emend. Safni et al.; Synonym: *Burkholderia solanacearum* (Smith 1896) Yabuuchi et al.; *Pseudomonas solanacearum* (Smith 1896) Smith 1914; *Xanthomonas solanacearum* (Smith) Dowson 1943); *Erwinia solanacearum* (Smith) Holland 1920 and many more.

Phylotype: Phylotype II

Name: *Ralstonia psuedosolanacearum* Safni, Cleenwerck, deVos, Fegan, Sly & Kappler; Synonym: *Burkholderia solanacearum* (Smith 1896) Yabuuchi et al.; *Pseudomonas solanacearum* (Smith 1896) Smith 1914; *Xanthomonas solanacearum* (Smith) Dowson 1943); *Erwinia solanacearum* (Smith) Holland 1920 and many more.

Phylotype: Phylotypes I and III

Name: *Ralstonia syzygii* (Roberts et al. 1990) Vaneechoutte et al. 2004 emend. Safni et al., 2014; Synonym: *Burkholderia solanacearum* (Smith 1896) Yabuuchi et al.; *Pseudomonas solanacearum* (Smith 1896) Smith 1914; *Xanthomonas solanacearum* (Smith) Dowson 1943); *Erwinia solanacearum* (Smith) Holland 1920 and many more.

Phylotype: Phylotype IV

Taxonomic position (EPPO 2023)

Domain – Eukaryota
Kingdom – Bacteria
Phylum – Proteobacteria
Class – Betaproeoobacteria
Order – Burkholderiales
Family – Burkholderiaceae
Genus – Ralstonia
Species – Ralstonia solanacearum, Ralstonia pseudosolanacearum, Ralstonia syzygii

Detection

Symptom

The bacterium causes rapid wilting of leaves and stems. Plant turns yellow and necrotic as the disease progresses. Brown discolouration of stems above the soil line may become visible. Slimy yellowish bacterial ooze is visible on cut surfaces of the plant.

In potato, oozes may be visible on the eyes and stolons. A brownish ring is visible along the vascular ring when tubers are cut where bacterial ooze appear (Figure 1).

Bacterial streaming test

Place a cut stem or tuber in water and observe for a milky thread-like bacterial ooze streaming from the cut plant into the water. Caution: do not mistake the cloudy exudates released immediately when a cut potato tuber is placed in water for bacterial ooze– this is just starch particle being released, wait for a few a sec to 1 min to observe the milky thread-like bacterial ooze.

Bacterial streaming test is however not confirmatory test.

Figure 1. Symptoms on cut tubers: (A) brown discolouration; (B) yellowish bacterial ooze (photo: N.Om).

Immunofluorescence test

Specific antibodies are available to detect the bacteria. The following field test kits and immunoflurescence (EPPO 2022):

- Pocket diagnostic (PD51119) with monoclonal antibody 9Y against *R. solanacearum* (Phylotype II).
- ImmunoStrip (ISK 33900) with monoclonal antibody Ps- 1 against *R. pseudosolanacearum.*
- Loewe Biochemica, Sauerlach, DE (07356).
- Plant Print Diagnostics S. L., Valencia, ES (1546-H/IVIA).
- Primer Diagnostics, Wageningen, NL (B-RsoI_C).

Identification

Identification can be done both through morphological and molecular methods.

Morphological methods

Isolation from symptomatic plant samples

- Take a section of symptomatic tissue (discoloured tissue but not totally rotten or decayed) or take the bacterial ooze and suspend in a small volume e.g., 5 mL of sterile distilled or RO water for 5-10 min. Plant tissue may be mashed before placing in the water.
- Place 50-100 µL of the bacterial suspension and spread it on general nutrient agar such as potato dextrose agar (PDA), nutrient (NA), yeast peptone glucose agar (YPGA), sucrose peptone agar (SPA) or on semi-selective mSMSA/Sequeira medium (Table 1).
- Incubate the plates at 28C for 2-6 days.
- Pick single colony and subculture on nutrient agar or semi-selctive media for storage or further identification work using microscopy and molecular methods.

Sampling and isolation from asymptomatic plant samples

Refer EPPO (2022) 'PM 7/21 (3) Ralstonia solanacearum, R. pseudosolanacearum and R. syzygii (Ralstonia solanacearum species complex)'.

Colony characteristics

- On general nutrient media, colonies of virulent strains develop as flat, irregular and fluidal colonies which are cream to white in colour. Small non-fluidal colonies are that of non-virulent strains.
- On mSMSA/Sequeria medium, virulent forms produce colonies with blood red whorls while those of non-virulent are small, round and non-fluidal and are entirely deep red in colour.

Molecular methods

Several molecular methods involving conventional PCR tests, TaqMan real-time PCR, and loop-mediated isothermal amplification (LAMP) are available. DNA sequencing is performed for accurate identification of phylotypes and sequevars using 16S rDNA, egl, mutS and hrB barcodes.

Laboratory requirements

- 2.0, 20, 200, and 1000 µL sterile pipette tips (prefer barrier tips)
- Microcentrifuge
- Microcentrifuge tubes 1.5 and 2.0 mL.
- Benchtop vortexer.
- 0.2 mL PCR tubes
- Thermocycler
- Gel tray with suitable comb/s, electrophoresis tank and powerpack
- UV transilluminator; Camera/gel documentation system
- PCR reagents
- Running buffer, 0.5 - 1x TBE (see NPPC protocol on buffer preparation)
- Agarose
- DNA ladder
- DNA staining agent (e.g., GelRed, avoid use of ethidium bromide) & Gel loading dye

DNA extraction from plant tissue

Refer specific DNA extraction methods provided by the manufacturer if using a commercial extraction kit (e.g., Bioline, Puregene, Qiagen, Sigma, etc.). Refer NPPC protocol on DNA extraction from plant tissue.

DNA extraction from colonies

- Follow manufacturer's protocol if using commercial kits
- Isolate sample on culture media and incubate for up to 7 days.
- Once colonies are formed, suspend one colony in 100 µL of PCR water.
- Heat at 95°C or 100°C for a minimum of 10 min. A freezing step before heating may be performed.
- Store lysate at -20°C till further use.

For more details refer EPPO (2022) 'PM 7/21 (3) Ralstonia solanacearum, R. pseudosolanacearum and R. syzygii (Ralstonia solanacearum species complex)'.

Conventional PCR

Prepare PCR mix reaction as per the protocol received with the DNA Taq DNA polymerase or use the protocol in Table 2.

Use the primer pairs and PCR conditions in Table 3. Run the PCR product in a TBE gel. After the gel electrophoresis, PCR product should produce a band at 320bp, 550bp and 500bp for actin, beta-tubulin and calmodulin gene region respectively. The PCR products should be purified using PCR Purification Kit by following manufacturer's protocol. The purified products should submitted to DNA sequencing facilities (Macrogen Inc. or others). The DNA sequence data is BLAST analyzed in NCBI and compared with reference sequence data listed in Table 3 for confirmation.

Table 1. Artificial media (EPPO, 2022).

Generic media			
Nutrient Agar (NA)		**Sucrose peptone agar (SPA)**	
Peptone	5.0 g	Sucrose	20.0 g
Yeast extract	3.0 g	Peptone	5.0 g
NaCl	0.5 g	K_2HPO_4	7.0 g
Microbiological grade agar	15.0 g	Microbiological grade agar	12.0 g
Distilled water	1.0 L	Distilled water	1.0 L
Yeast peptone glucose agar			
Yeast extract	5.0 g		
Bacto peptone	5.0 g		
Glucose	10.0 g		
Microbiological grade agar	15.0g		
Distilled water	1.0 L		
Adjust pH to 7.2			
Semi-selective media			
Modified semi-selective medium from South Africa (mSMSA)			
Bacto peptone	10.0 g	Autoclave and cool to 45-50°C and add the following:	
Glycerol	5 mL	2,3,5-triphenyl-2H-tetrazolium chloride	0.050 g
Bacto Agar	15.0 g	Crystal violet	0.005 g
Casamino acid	1.0 g	Chloramphenicol (water soluble)	0.005 g
Yeast extract	1.0g	Penicllin G (benzylpenicillin sodium salt)	825 U
Deionized distilled water	1 L	Polymyxin B (sulphate salt)	600 000 U
		Bacitracin	1250 U
Final pH should be around 6.5			

Table 2. PCR mix preparation

Reagents	µl/reaction	Final concentration
PCR water	12.375	
10x PCR mix (without MgCl2)	2.5µl	1x (2mm MgCl2)
MgCl2 (25mM)	0.5	
dNTP Mix (10mM each)	0.125 µL	0.1 mMeach
RS-1-F (10µM)	2 µL	0.8 µM
RS-1-R (10µM) or RS-3-R	2 µL	0.8 µM
NS-5-F (10µM)	0.15 µL	0.06 µM
NS-6-R(10µM)	0.15 µL	0.06 µM
Taq (DNA polymerase (5µ/µl)	0.2 µL	1 U
DNA template	5 µL	
Total reaction volume	25 µL	

Note: if BSA is used adjust the volume of PCR water to obtain the final volume of 25 µL

Table 3: Primer pair for conventional PCR to detect *R. solanacearum* and *R. pseudosolanacearum.*

Species/strain	Amplicon	Primer pair	Primer Sequence
R. Solanacearum (Phylotype II)	718 bp	RS-1-F	5′-ACTAACGAAGCAGAGATGCATTA-3′
		RS-1-R	5′-CCCAGTCACGGCAGAGACT-3′
R. pseudosolanacearum (Phylotype I)	716 bp	RS-1-F	5′-ACTAACGAAGCAGAGATGCATTA-3′
		RS-3-R	5′-TTCACGGCAAGATCGCTC-3′
Both. Use as internal control	310 bp	NS-5-F	5′-AACTTAAAGGAATTGACGGAAG-3
		NS-6-R	5′-GCATCACAGACCTGTTATTGCCTC-3′.

Multiplex the above primers. Include PCR mix control and water control, and positive control if available. Save positive DNA samples for use as positive control in future tests.

PCR conditions for both sets of primers:

PCR conditions: an initial 5-min incubation at 95°C followed by 35 cycles of 30 s at 95°C, 30 s at 58°C and 45 s at 72°C, followed by a final extension of 5 min at 72°C. Hold at 10°C.

PCR result interpretation:

- The test is considered positive if a band of 718 bp (RS-1-F and RS-1-R), 716 bp (RS-1-F and RS-3-R) and 310 bp (NS-5-F and NS-6-R) is visualized.
- The test is considered negative if no band or a band of a different size than expected is visualized.
- Repeat the tests if any of the controls did not work.

Real-time PCR

Use the real-time reaction mix in Table 4 and primer pairs in Table 5 to conduct real-time PCR.

Table 4. Real-time PCR mix preparation.

Reagents	**µL/reaction**	**Final concentration**
PCR water	12. 875	
10x PCR buffer (without MgCl2)	2.5 µL	1x (2mm MgCl2)
MgCl2 (25mM)	3.5 µL	3.5 mM
dNTP Mix (10mM each)	2 µL	0.2 mM each
Forward primer mix of RS-I-F, B2-I-F, COX-F (10µM)	0.75 µL	0.3 µM
Reverse primer mix: RS-I-R, B2-II-R, COX-R (10µM each)	0.75 µL	0.3 µM
Probe mix of RS-P, B2-P, COX-P, RSP-55T	0.5 µL	0.1 µM
Taq (DNA polymerase (5µ/µl)	0.125 µL	1 U
DNA template	2 µL	
Total reaction volume	**25 µL**	

OR

Table 4. Real-time PCR mix preparation.

Reagents	**µL/reaction**	**Final concentration**
PCR water	12. 875	
10x PCR buffer (without MgCl2)	2.5 µL	1x (2mm MgCl2)
MgCl2 (25mM)	3.5 µL	3.5 mM
dNTP Mix (10mM each)	2 µL	0.2 mM each
Forward primer: RS-I-F, (10µM)	0.25 µL	0.3 µM
Forward primer: B2-I-F,	0.25 µL	0.3 µM
Forward primer: COX-F	0.25 µL	0.3 µM
Reverse primer: RS-I-R (10µM)	0.25 µL	0.3 µM
Reverse primer: B2-II-R (10µM)	0.25 µL	0.3 µM
Reverse primer: COX-R (10µM)	0.25 µL	0.3 µM
Probe: RS-P	0.125 µL	0.1 µM
Probe: B2-P	0.125 µL	0.1 µM
Probe: COX-P)	0.125 µL	0.1 µM
Probe: RSP-55T	0.125 µL	0.1 µM
Taq (DNA polymerase (5µ/µl)	0.125 µL	1 U
DNA template	2 µL	
Total reaction volume	25 µL	

Table 5. Primers and probes for TaqMan real-time PCR.

Species/strain	Primer pair	Primer Sequence
The primer probes target specific 16S rRNA gene within R. solanacearum, R. pseudosolanacearum and R. syzygii strains.	RS-I-F	5′-GCATGCCTTACACATGCAAGTC-3′
	RS-II-R	5′-GGCACGTTCCGATGTATTACTCA-3′
	B2-I-F	5′-TGGCGCACTGCACTCAAC-3′
	B2-II-R	5′-AATCACATGCAATTCGCCTACG-3′
	COX-F	5′-CGTCGCATTCCAGATTATCCA-3′
	COX-R	5′-CAACTACGGATATATAAGAGCCAAAACTG-3′ (COX F/R and COX P target plant cytochrome oxidase if plants samples are used).
Improve specificity with the following		
RS-I-FmAK		5′-CATGCCTTACACATGCAAGTC-3′
RS-II-RmAK		5′-CACGTTCCGATGTATTACTCA-3′
RS-P (probe)		5′-[FAM]-AGCTTGCTACCTGCCGGCGAGTG-[TAMRA]-3″
B2-P (probe)		5′-[VIC]-TTCAAGCCGAACACCTGCTGCAAG-[TAMRA]-3′
COX-P (probe)		5′-[VIC]-TGCTTACGCTGGATGGAATGCCCT-[TAMRA]-3′
RSP-55T* (probe)		5′-[FAM]-AGCTTGCTACCTGCCGG-[NFQ-MGB]-3'

*Modification of the RS-P probe. This probe help in reducing false-positive results.

PCR controls must be used:

- Positive isolation control (PIC) – which is disease plant (if available) should be used.
- Negative isolation control (NIC) which is the buffer 50mM phosphate buffer, or a disease free plant extract used for isolation of bacterial from plant tissue.
- Positive control (PC) which is positive DNA extract
- Negative control or non-tempalate control (NTC) – which usually PCR water instead of DNA,

Result interpretation

- The PIC and PC, should show exponential amplification curves for both primers and probe channels.
- The NIC and NTC should have any amplification curves for both primer and probe channels.

When these conditions are met

- A test will be considered positive if it produces an exponential amplification curve.
- A test will be considered negative if it does not produce an amplification curve or if it produces a curve which is not exponential.
- Tests should be repeated if any contradictory or unclear results are obtained.

Reference

EPPO (2022). PM 7/21(3) Ra*lstonia solanacearum*, *R. pseudosolanacearum* and *R. syzygii* (*Ralstonia solanacearum* species complex). EPPO Bulletin (52): 225–261.

NPPC protocol on DNA extraction from Plant, Insect, Fungi and Bacteria sample using CTAB method

(Updated on August, 2024, S Chophel)

NPPC protocol on DNA extraction from plant sample using CTAB method (modified Doyle and Doyle, 1990)

Materials Needed:

- Sample
- Homogenizer
- FastPrep
- Beating beads
- Weighing balance
- 1.5 ml microcentrifuge tubes
- Sterile loop or pipette tips
- Pipettes and sterilised tips ((10µl, 200µl, 1000µl)
- Centrifuge
- Vortex mixer
- Incubator or water bath (55°C)

Solutions:

- CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 1% β-mercaptoethanol)
- Chloroform alcohol (24:1) (Chloroform: iso-amyl alcohol, 24:1)
- Isopropanol
- 70% ethanol
- Nuclease-free water
- Sterile distilled (molecular grade) water or TE buffer

Procedure:

1. Label all tubes and plastic pouch before starting extraction: two sets of 2 mL tubes and one set of 1.5 mL tube. Turn on the water bath with the temperature set at 65°C.
2. Wash samples thoroughly (or if using barks: place the barks in a petri dish with distilled water, clean the barks by scrapping off all dead tissues on the outside very lightly with a scalpel, then rinse with distilled water two times). *Include an extraction control comprising of a disease free plant (same species if available if not any other plant).*
3. Blot dry with tissue paper/paper towel.
4. Using disposable blades, cut mid ribs (or cut the barks) and transfer (0.5 g) in the extraction pouch.
5. Add 3–3.5 mL of CTAB (add 20 µL of β-mercapto ethanol per 10 mL of CTAB just before use to each pouch. S
6. Grind the samples with the homogenizer at 3000 rpm until no tissue are visible. Homogenization can also be done using Fastprep instrument.

(If using FastPrep, grind plant tissue using liquid nitrogen before placing CTAB: place 0.5g tissue in 2mL tube (special tube with screw cap), place sterile 6 mm diameter beating beads, dip tubes in liquid nitrogen for 10 sec then place frozen tubes in the FastPrep instrument and secure well. Pulse following FastPrep protocol. Once tissue is ground to powder, add CTAB filling the tube to 1.9 mL. Then proceed to step 8 below).

7. Transfer all extracts into a 2 mL tube with a pastuer pipette.
8. Place all the tubes in a floater and incubate in the water bath for 30 min at 65°C (shake the tubes by inverting the tubes gently after 15 min).
9. Centrifuge for 5 min at 3000 rpm (~8000 x g) and 20°C.
10. Transfer 1 mL of the supernatant into another set of 2 mL tubes.
11. Add 1 mL of chloroform:Isoamyl alcohol (24/1) to each tube. Shake by inverting the tubes a few times.
12. Centrifuge at 20°C and 14000 rpm (~16100 x g) for 5 min.
13. Transfer ~0.850 to 0.9 mL (usually it is easier to transfer 850 µL without sucking the lower phase of the liquid) of the supernatant to 1.5 mL tube. Take care not to take in any chloroform from the bottom – if this happens, put back everything in the original tube and centrifuge again.
14. Add 0.54 mL (540 µL) of Isopropanol to each tube. (Isopropanol should be stored at −20°C).
15. Precipitate at −20°C for 1 hr if to be continued on the same day or at 4°C overnight if to be continued on the next day.
16. Centrifuge at 4°C and 14000 rpm for 20 min.
17. Discard the supernatant.
18. Re-suspend the pellet in 1mL of 70% ethanol.
19. Vortex slightly/mix by hand for few seconds by inverting.
20. Centrifuge at 20°C and 14000 rpm for 5 min.
21. Discard the supernatant.
22. Re-suspend the pellet in 95% ethanol.
23. Vortex slightly or hand mix by inverting.
24. Centrifuge at 20°C and 14000 rpm for 5 min.
25. Discard the supernatant. Carefully siphon off the remaining supernatant with a smaller calibrated pipette (20-200 µL). Discard tip for each sample.
26. Leave open the tubes for 30 min to 45 min on bench top.
27. Re-suspend the pellet in 80–100 µL of PCR water (or sterile distilled water) or in 1x TE buffer for long term storage or if transporting.
28. Store DNA at −20°C till further use.

NPPC protocol for DNA extraction from insect (psyllid) using CTAB method

1. Although single adult psyllid has been used to test for HLB pathogen, in our protocol we use groups of five psyllids (adults or 4–5th instar nymphs) for DNA extraction. If fewer psyllids are available, then use whatever number (> 1) is available but decrease the DNA elution volume to 20 µL.
2. For psyllids preserved in ethanol, dry the psyllid completely to let the ethanol evaporate. This can be achieved by placing the psyllids on clean paper towel using a fine tip brush. Then pick the desired number e.g., five adult psyllids, into each 1.5 mL tubes. Leave the tube open (in the laminar hood) for 10 min before adding extraction buffer. *Include extraction control using a disease free psyllid if available or another insect that is not a vector of CLaf/CLam/CLas, e.g., house fly.*
3. In each 1.5 mL tube containing psyllids, add 300 µL CTAB buffer and crush with a sterile plastic pestle.
4. Once the psyllids are macerated, incubate for 30 minutes at 65°C (shake the tubes by inverting the tubes gently after 15 min).
5. Centrifuge for 5 minutes at 3000 rpm (~8000 x g) at 20°C.
6. Transfer 150 µL of the supernatant into another set of 2 mL tubes.
7. Add 150 µL of chloroform: Isoamyl alcohol (24/1) to each tube. Shake by inverting the tubes a few times.
8. Centrifuge at 20°C and 14000 rpm (~16100 x g) for 5 min.
9. Transfer 120 µL of the supernatant to 1.5 mL tube. Take care not to take in any chloroform from the bottom- if this happens, put back everything in the original tube and centrifuge again.
10. Add 80 µL of cold Isopropanol to each tube. (Isopropanol should be stored at −20°C).
11. Precipitate at −20°C for 1 hr if to be continued on the same day or at 4°C overnight if to be continued on the next day.
12. Centrifuge at 4°C and 14000 rpm for 20 min.
13. Discard the supernatant.
14. Re-suspend the pellet in 200 µL of 70% ethanol.
15. Vortex slightly/mix by hand for few seconds by inverting.
16. Centrifuge at 20°C and 14000 rpm for 5 min.
17. Discard the supernatant.
18. Re-suspend the pellet in 200 µL 95% ethanol.
19. Vortex slightly or hand mix by inverting.
20. Centrifuge at 20°C and 14000 rpm for 5 min.
21. Discard the supernatant. Carefully siphon off the remaining supernatant with a smaller calibrated pipette (20–200 µL). Discard tip for each sample.
22. Leave open the tubes for 30 min to 45 min on bench top.
23. Re-suspend the pellet in 30 µL of PCR water (or sterile distilled water) or in 1x TE buffer for long term storage.
24. Store DNA at −20°C.

DNA extraction from insect (psyllids) using REDExtract-N-Amp™ Tissue (or plant) PCR Kit from Sigma

The protocol using this kit is modified as below depending on the number of psyllids used per extraction.

1. Set water bath to 95°C before proceeding
2. Place psyllids in 1.5 mL tube as described in the CTAB method.
3. For extraction from single adult psyllid, add 10 µL of the extraction buffer and crush the psyllid with blunt 20–200 µL pipette tip (flame the tip and press against the cap of the sample tube to make it blunt).
4. Incubate at 95°C for 10 min.
5. Remove from the water bath and add 10 µL dilution buffer.
6. Store the extract at −20°C till further use.
7. For extractions from 2–3 adults add 15 µL of each of extraction and dilution buffer.
8. For extraction from five adults, add 25 µL of each buffer.
9. For nymphs, use 10 µL and 15 µL of each buffer for 2–3 and five nymphs respectively.

NPPC protocol on DNA extraction from fungi and fungi-like organism using CTAB method

Materials

- Cultures
- 1.5 sterile tubes
- 2ml beating tubes (~2mm)
- Fast prep
- CTAB buffer
- 24.1 chloroform: isoamyl alcohol
- 70-90% ethanol (molecular grade)
- Isopropanol
- 1X TE buffer
- Water bath
- Micro pipette & tips (10µl, 200µl, 1000µl)

Procedure

1. Obtain two 7days old cultures (e.g., Phytophthora spp. grown on CA or Trichoderma sp. on CA/PDA), when the mycelia have covered the surface of the plate.
2. Add 1ml sterile distilled water to the edge of the culture plate.
3. Scrape the fungal mycelia from the surface of the agar with a sterile loop (avoid scraping agar).
4. Transfer to 2ml beating tube or 2ml PCR tubes using a Pasteur pipette.
5. Add 1ml more to the agar plate and scrape then add to the 2ml beating tube to make up to the 2ml mark.
6. Centrifuge at 14000 (…g) rpm @4°C for 20 minutes
7. Discard the supernatant and retain the pellet of mycelia
8. Place two ~2-2.6mm beating beads in each tube
9. Add 300µl of CTAB buffer (with mercaptoethanol) to each beating tube with mycelia and beating beads.
10. Grind using FastPrep @ 4/s for 20 second.
11. Centrifuge @ 14000 rpm for 5 minutes
12. Adjust final volume of CTAB to 1ml in the tube, mix well by flicking the tubes and dislodge the pellets from the bottom/side of the tube
13. Incubate at 65°C for 30 minutes. Shake by flicking the tubes after 15 minutes.
14. Centrifuge for 5 minutes at 3000 rpm
15. Add 500µl of supernatant to 1.5ml tube.
16. Transfer 500µl of chloroform: isoamyl alcohol (24:1). Shake the tubes by inverting the tubes a couple of times.
17. Centrifuge at 20°C and 14000 (…g) rpm for 5 minutes.
18. Transfer the 400µl of the supernatant to 1.5ml tube.
19. Add 240µl of cold Isopropanol (stored at -20°C)
20. Precipitate at -20°C for 30 minutes or at 4°C overnight.
21. Centrifuge at 4°C and 14000 (….g) rpm for 20 minutes
22. Discard the supernatant

23. Resuspend the pellet in 1ml 70% ethanol
24. Gently vortex for few seconds
25. Centrifuge 20°C and 14000 rpm for 5 minutes
26. Discard the supernatant
27. Repeat steps 23 to 25 for 3 times
28. Discard the supernatant. Gently siphon off the remaining supernatant with 20-200 µl
29. Air dry pellets. Leave tubes open for about 30 to 45 minutes
30. Add 30µlof 1X TE buffer and store DNA at - -20°C till further use.

NPPC protocol on DNA extraction from bacterial colony using CTAB method (adapted from Moore et al., 2004)

Materials Needed:

- Bacterial culture grown on PDA
- 1.5 ml microcentrifuge tubes
- Sterile loop or pipette tips
- Pipettes and sterilised tips ((10µl, 200µl, 1000µl)
- Centrifuge
- Vortex mixer
- Incubator or water bath (55°C)

Reagent Solutions:

- CTAB extraction buffer (2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, 1% β-mercaptoethanol)
- Proteinase K ((20 mg/ ml): Dissolve 100 mg proteinase K in 5 ml sterile distilled water. Keep at – 20 ºC)
- Chloroform alcohol (24:1) (Chloroform: iso-amyl alcohol, 24:1)
- Isopropanol
- 70% ethanol
- Nuclease-free water
- Sterile distilled (molecular grade) water or TE buffer

Procedure:

Sample Collection

1. Use a sterile loop or pipette tip to collect a small amount of bacterial growth from the PDA plate. (Should weigh approximately 0.1g taken from 1 to 5 colonies)
2. Transfer the bacterial sample into a 1.5 ml microcentrifuge tube containing 500 µl of sterile water or TE buffer and dissolve completely
3. Vortex the tube vigorously to suspend the bacterial cells.

Cell Lysis

4. Add 500 µl of CTAB extraction buffer to the tube
5. Add 20 µl of Proteinase K (20 mg/ml) to the mixture.
6. Mix thoroughly by vortexing.

Incubation

7. Incubate the tube at 55°C for 30 minutes. Shake by flicking the tubes after 15 minutes (This step facilitates the lysis of bacterial cells and the release of DNA)
8. Transfer 500 µl of the supernatant to 1.5ml microcentrifuge tube.
9. Add 500 µl of chloroform: isoamyl alcohol (24:1) to the tube. Shake the tubes by inverting the tubes a couple of times.
10. Centrifuge the tube at 12,000 rpm for 10 minutes at 20°C.

11. Carefully transfer the upper aqueous phase (400 µl) to a new 1.5 ml microcentrifuge tube, avoiding the interface.

DNA Precipitation:

12. Add an equal volume of isopropanol (approximately 400 µl) to the aqueous phase to precipitate the DNA.
13. Mix gently by inverting the tube several times.
14. Precipitate (incubate) at -20°C for at least 30 minutes or at 4°C overnight (to ensure DNA precipitation)

DNA Pelleting:

15. Centrifuge the tube at 12,000 rpm for 10 minutes at 4°C.
16. Carefully discard the supernatant without disturbing the DNA pellet.
17. Wash the pellet with 500 µl of 70% ethanol to remove any residual CTAB and salts.
18. Centrifuge again at 12,000 rpm for 5 minutes at 4°C.
19. Discard the supernatant and air-dry the DNA pellet for about 30 to 45 minutes (do not over dry)

DNA Resuspension:

20. Resuspend the DNA pellet in 50 µl of TE buffer.
21. Store the DNA at -20°C until ready for PCR analysis or other applications.

NPPC protocol on RNA extraction from plant sample using CTAB method (modified Doyle and Doyle, 1987; Aladizgeh et al 2023)

Materials Needed:

- Sample
- Homogenizer
- FastPrep
- Beating beads
- Weighing balance
- 2ml /1.5 ml microcentrifuge tubes
- Pipettes and sterilised tips ((10µl, 200µl, 1000µl)
- Centrifuge
- Vortex mixer
- Incubator or water bath

Reagent Solutions:

- Lysis buffer / CTAB extraction buffer (2% (v/v) CTAB, 100 mM Tris-HCl, 25 mM EDTA, 2 M NaCl)
- 2.5%m/v PVP-40 (Polyvinylpyrrolidone
- 2% β-mercaptoethanol)
- Chloroform: isoamyl alcohol (24:1 v/v)
- 2.5 M lithium chloride (LiCl) or Isopropanol
- 70% ethanol
- Nuclease-free water
- Sterile distilled (molecular grade) water or TE buffer

Procedure:

1. Label all tubes and plastic pouch before starting extraction: two sets of 2 ml tubes and one set of 1.5 mL tube. Turn on the water bath with the temperature set at 65°C.
2. Grind 100 mg (0.1 g) plant tissues (e.g. leaf, shoot, root) in 1000 µl of prewarmed (at 65 °C) lysis buffer.
3. Transfer crushed sample to 2 ml PCR or eppendorf tube and add 10 µl β-mercaptoethanol and vortexed for 1 min.
4. Incubated at 65 °C in a water bath for 10 minutes (shake the tubes by inverting the tubes gently after 5 minutes).
5. Centrifuge at 13000 rpm for 4 min at room temperature (~ 20 °C – 22 °C).
6. Collect supernatant 900 µl into 2 ml PCR tube.
7. Add an equal volume (900 µl) of chloroform: isoamyl alcohol (24:1) and vortexed for 1 minutes.
8. Centrifuge at 13000 rpm for 10 minutes at 20 °C.
9. Transfer upper aqueous phase (600 µl) to 1.5 ml PCR tube gently, without disrupting the solid and organic phases.
10. Add an equal volume (600 µl) of cold Isopropanol to each tube. (Isopropanol should be stored at −20°C).

(Or transfer 600 µl into tube which half the amount (300 µl) of lithium chloride (7.5M) was added, and stored at −20 °C for 15–20 min.).

11. Precipitate at −20°C for 1 hr if to be continued on the same day or at 4°C overnight if to be continued on the next day.
12. Centrifuge samples at 13000 rpm for 30 min at 4 °C.
13. Discard the supernatant and resuspend the pellet with 500 µl ice-cold ethanol (80%v/v).
14. Vortex slightly/mix by hand for few seconds by inverting.
15. Centrifuge pellets at 13000 rpm for 5 min at 4 °C.
16. Again, decant the tube and let the pellet air-dry for approximately 30–40 min.
17. Resuspend the pellet in 50 µl of RNase-free water or nuclease free water.
18. Store RNA at −20°C till further use.

Reference

Aladizgeh. F.M., Jabbari. L., Khayam., N. R, Aalami. A., Atwell.B.J., Haynes. P.A.2023. A universal protocol for high-quality DNA and RNA isolation from diverse plant species. PLoS ONE 18(12): e0295852. https://doi.org/10.1371/journal.pone.0295852

NPPC protocol on Gram Staining of bacteria (modified from Gephardt et al., 1981)

Principle:

Gram staining is a diagnostic test that gives an early indication of potential bacteria through visualization of the bacteria. The Gram stain helps to differentiate the organism, whether it is gram-positive or gram-negative. Gram-positive bacteria appear purple in colour and gram-negative bacteria appear pink. Differences of Gram-positive and Gram-negative bacteria arise from cell wall composition; Gram-positive bacteria have a thick peptidoglycan layer with teichoic acids, making them resistant to decolorization.

The Gram staining procedure involves four basic steps:

1. The bacteria are first stained with the basic dye **crystal violet**. Both Gram-positive and Gram-negative bacteria become directly stained and appear purple after this step.

2. The bacteria are then treated with **Gram's iodine solution**. This allows the stain to be retained better by forming an insoluble crystal violet-iodine complex. Both Gram-positive and Gram-negative bacteria remain purple after this step.

3. **Gram's decolorizer**, a mixture of **ethyl alcohol and acetone**, is then added. This is the differential step. Gram-positive bacteria retain the crystal violet-iodine complex while Gram-negative are decolorized.

4. Finally, the counterstain **safranin** (also a basic dye) is applied. Since the Gram-positive bacteria are already stained purple, they are not affected by the counterstain. Gram-negative bacteria, which are now colourless, become directly stained by the safranin. Thus, Gram-positive appear purple, and Gram-negative appear pink.

Interpretation

Gram-positive bacteria (thick layer of peptidoglycan) - **stains violet /purple**
Gram-negative bacteria (thin layer of peptidoglycan and high lipid content) –**stains red/pink**

Materials

- Culture containing bacteria (18 to 24 hours old)
- Bunsen burner
- Compound microscope
- Slides and coverslip
- Inoculation needle or Inoculating loop
- Immersion oil
- Gram staining kit (Reagents)

Reagents needed for Gram staining include:

- Crystal violet (primary stain)

- Gram's iodine solution (the mordant)
- Acetone/ethanol (50:50 v: v) (the decolorizer)
- **Safranin** solution (the counterstain/secondary stain)
- Water

The procedure of Gram Staining

1. **Preparing Bacterial smear**

1. Take a clean microscopic slide
2. Place a drop of sterilized distilled water on the microscopic glass slide with the help of an inoculation loop.
3. Sterilize the inoculating loop by flaming and transfer a small portion of the bacterial colony from the bacterial culture plate and emulsify it with a drop of water on the glass slide. (If culture is on a petri dish or slant, place a drop of water in the middle of the glass slide and using a sterile loop, transfer a small amount of colony and suspend with the water drop).
4. After emulsifying spread the smear to a size of about 1cm sq., to form a thin film.
5. After spreading allow it to air dry for 2-3 minutes.

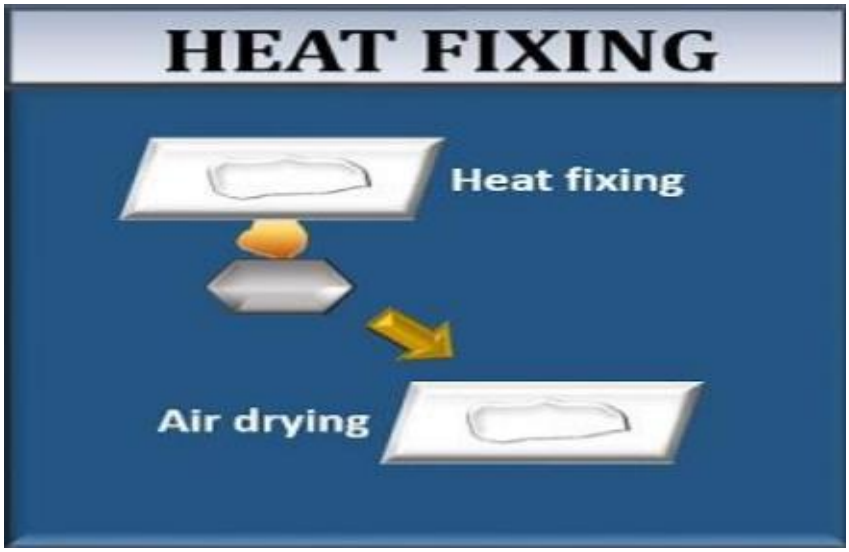
2. Heat Fixing

After smear preparation, move the prepared slides over the Bunsen burners flame at least three times. Then, allow the slide to air dry.

• Heat fixing helps in the fixation of a specimen to the glass slide.

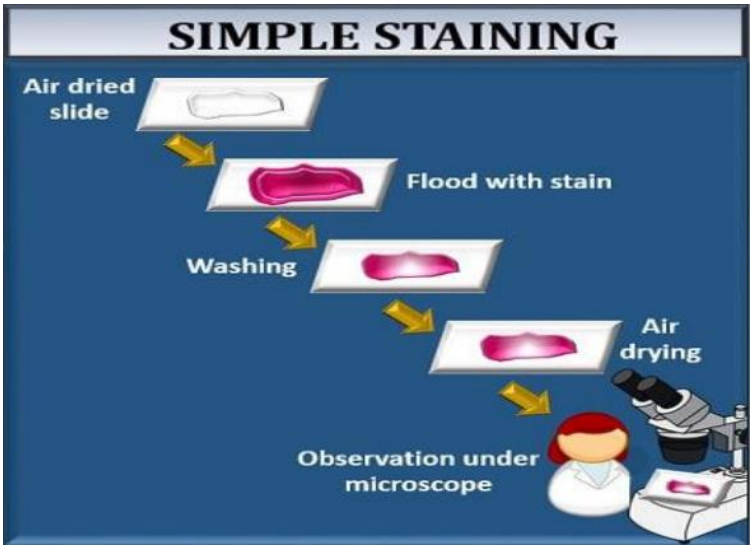
• Heat fixing helps the stain to penetrate the smear.

(Slide must be moved up and down or circularly over the flame to prevent from overheating)



3. Staining the Bacterial smear

1. Add crystal violet staining solution over the smear and allow it to stand for 1 minute.
2. Wash slide in a gentle and indirect stream of distilled water for 2 seconds.
3. Flood slide with the mordant: Gram's iodine. wait for 1 minute.
4. Wash slide in a gentle and indirect stream of distilled water for 2 seconds.
5. Flood slide with a decolorizing agent (Acetone-alcohol decolourizer). Wait 10-15 seconds or add drop by drop to slide until decolorizing agent running from the slide runs clear.
6. Flood slide with a counterstain, safranin. Wait 30 seconds to 1 minute.
7. Wash slide in a gentile and indirect stream of distilled water until no colour appears in the effluent and then blot dry with absorbent paper.
8. Observe the results of the staining procedure under oil immersion (100x) using a bright field microscope.



Reagent preparation if the Gram staining kits are not available from solid stock or powder

1. **Crystal Violet Preparation**
    - For preparing solution A (Crystal Violet stock solution), add 20 gm of 85% crystal violet dye in 100 ml ethanol (95%) and dissolve by mixing thoroughly.
    - For preparing solution B (Oxalate Stock solution), add 1 gm ammonium oxalate in 100 ml distilled water and dissolve by mixing thoroughly.
    - For preparing the working solution, add 1 ml of crystal violet stock solution in 10 ml distilled water and add 40 ml of oxalate stock solution.
    - Let the solution sit for 24 hours at room temperature
    - Store the solution in a dark bottle for use.
2. **Gram's Iodine Preparation**
    - Dissolve 1 gm of Iodine, and 2 gm of potassium iodide in 300 ml distilled water.
    - Mix properly till the iodine dissolve and keep the solution in a dark bottle.
3. **Decolorizing Solution**
    - Mix 50 ml of acetone with 50 ml of 95% ethanol
4. **Safranin**
    - Mix 2.5 gm of safranin-O in 100 ml of 95% ethanol
    - Mix 10 ml of the above solution with 90 ml of distilled water to prepare a working solution
5. **Carbol-fuchsin**
    - Dissolve 3 gm of basic fuchsin in 100 ml of 95% ethanol
    - Mix 5 ml liquid phenol with 95 ml of distilled water to prepare 5% phenol solution
    - Mix 10 ml of basic fuchsin solution with 100 ml of 5% phenol solution
    - Let the solution sit for 24 hours at room temperature
    - Store in a dark bottle

References

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Gephart, P., Costilow, R, N., Krieg, N.R., Murray, R.G.E., Nester, E.W., Phillips, G.B., and Wood, A.W. B 1981. Manual of Methods for General Bacteriology, ASM Press, Washington D.C. Gram. C.1884. Ueber die isolirte Färbung der Schizomyceten in Schnitt-und Trockenpräparaten. Fortschritte der Medcin, (2):185-189.

NPPC protocol on Buffer preparation

Remember to use a measuring cylinder to calibrate your flasks/bottles. Example; measure 500ml or 1000ml water with the measuring cylinder and pour to the empty flask that you are going to use for the final volume of 500ml/1 litre flask. Mark on the flask or the bottle where the volume has reached. This marking will help you to adjust your final volume whether it is 500ml or 1000ml after dissolving all solutes and other additives like HCL or NaOH.

1M Tris HCL for 500ml volume (Molecular weight of Tris = 121.1g/M)

1. Add 60.55 g of Tris in a 500ml or 1 litre flask
2. Then add 400ml of distilled water
3. Dissolve using magnetic stirrer
4. Measure pH with pH meter and adjust to pH 8 by adding HCL drop by drop and stir
5. Adjust final volume to 500ml
6. (if you add 121.1 g of Tris in 1L = 1M, so half amount for half volume)

Tris HCL*: Tris hydrochloride, is a buffering agent (acidic buffer) commonly used by molecular biologists to adjust the pH of a solution or stabilize the pH.*

0.5M of EDTA (MW = 372.24g/mol) pH 8 for 500ml

Here the concentration is 0.5M. if you add half amount of the molecular weight that is 186.12g in 500ml then you will get 1M. which means to achieve a 0.5M in 1L, you will need to add half the amount (372.24 divided by 2 = 185.12g). For 500ml of 0.5M, you will need to add half of 186.12g which is 93.06g.

1. Add 93.06g in a 1L flask
2. Add 400ml of distilled water and stir using magnetic stirrer. Add pellets/powder of NaOH to dissolve EDTA which otherwise will remain very compact. Add NaOH in smaller amount.
3. Once EDTA has dissolved (i.e. becomes a clear liquid), adjust pH by HCL to pH 8.
4. Adjust final volume to 500ml

Autoclave both Tris HCL and EDTA at 120°C for 15 minutes.

****EDTA:*** *Ethylenediamine tetra acetic acid, is a chelating agent that binds divalent metal ions such as calcium and magnesium. EDTA can be used to prevent degradation of DNA and RNA and to inactivate nucleases that require metal ions. EDTA can also be used to inactivate metal ion-requiring enzymes.*

50ml of 10X TE buffer

1. Take 44ml of sterile distilled water in a 15oml or 250 ml bottle (use sterilized bottles)
2. Add 5ml of 1M Tris HCl, and 1ml of 0.5M EDTA (use autoclaved ones)
3. Mix well
   It is better to add Tris HLC and EDTA to water

10ml of 1X TE buffer

1. Add 9ml of sterile distilled water
2. Add 1ml of 10X TE buffer
3. Mix well

TE: *Tris-EDTA, are use in maintaining the pH of the solution and solubilizing DNA or RNA while protecting the nucleic acids from enzymatic lysis. TE buffer is made up of Tris, a pH buffer and EDTA, a metal chelating ion. It is used in DNA extraction processes to lyse, wash and dissolve DNA. TE buffer is also used for DNA storage because of its ability to store DNA for a longer period of time without degrading it.*

CTAB (N-Cetyl-N, N, N-Trimethylammonium Bromide) for 1 litre volume

Warm some sterile distilled water I the water bath or on hot plate before preparing this.

1. Weigh and add 20g of CTAB and put in a 1 litre flask
2. Then add 82g of NaCl and 20g of PVP 10,000. (If the PVP is 40,000 or PV40, then the amount should be four times less.
3. Add 400ml of warm sterile distilled water (~50°C) (CTAB dissolves better in warm water)
4. Add 100ml of 1M Tris HCL (use the autoclaved one)
5. Add 40ml of 0.5M EDTA (use the autoclaved one)
6. Dissolve CTAB on hot plate at 50°C and stirring with a magnetic stirrer. (This takes time, it is better to prepared in the morning or late afternoon and keep CTAB stirring overnight if it takes too long to dissolve
7. Adjust final volume to 1 litre. **(Do not autoclave CTAB)**
8. Do not add β-mercapto to the stock of CTAB

PVP*: Polyvinylpyrrolidone,*

Chloroform / Isoamyl alcohol (24:1)

Add 1ml of isoamylic alcohol to 24ml of chloroform. Prepare bulk of 250ml by adding 10ml isoamylic alcohol to 240ml chloroform.

Ethanol 70% for final volume of 200ml (use molecular grade for extraction).

Use the other ethanol for cleaning purposes.

140ml ethanol (100% or 99%) plus 60ml sterilized distilled water.

Notes

1. Prepare fresh Tris-HCL and EDTA every six months. CTAB should be prepared one to two days before extraction and add β-mercapto just before dispensing into the extraction tubes or products.
2. Sterilize all glass wares used for CTAB and TE buffer.

General Laboratory operating procedures for NPPRL, NPPC (modified from Jonathan Klane, 2017)

(Prepared by: S Chophel, 2024)

(A principal cause of laboratory accidents is poor housekeeping)

General laboratory safety Practices

1. Be sure to read all fire alarm and lab safety symbols and signs and follow the instructions.
2. Ensure you are fully aware of your facility's/ building's evacuation procedures-where lab's exits and fire alarms are located.
3. Make sure you know where your lab's safety equipment (first aid kits, fire extinguishers, eye wash stations and safety showers).
4. Know emergency phone numbers to use to call for help in emergency.
5. Open flames should never be used in the laboratory.
6. Always work in properly ventilated areas.
7. Do not chew gum, drink, eat or apply lip balm or cosmetics while working in the lab.
8. Laboratory glassware should never be used as food or beverage containers.
9. If you are the last person to leave the lab, make sure to lock all the doors, switch off electrical appliances and turn off all ignition sources.
10. Never lift any glassware, solutions, or other types of apparatus above eye level.
11. Make sure you always follow the proper lab safety procedures for disposing of lab waste.
12. In the event of a chemical splashing into your eye(s) or on your skin, immediately flush the affected area(s) with running water for at least 20 minutes
13. Do not work alone in the lab.

General Housekeeping safety rules

1. Keep all work areas, and especially work benches, clear of clutter and obstructions.
2. Access to emergency equipment, showers, eyewash stations, and exits must never be blocked.
3. Coats, bags, and other personnel items must be stored in the proper area, not on the bench tops, or near chemicals or equipment that is in use.
4. Keep all aisles, hallways, and stairs clear of all chemicals and other obstructions; *never store or place chemicals on the floor*.
5. Promptly clean up all spills, including water spills and ice, and properly dispose of all spilled chemicals.
6. Keep drawers and cabinets closed to avoid accidents.
7. All work surfaces and floors should be cleaned regularly and keep work surfaces dry.
8. Promptly dispose/recycle packing materials and empty cartons.

Dress code safety rules

1. Always tie back hair that is chin-length or longer and as needed.
2. Make sure that loose clothing or dangling jewellery is removed, or avoid wearing it in the lab.
3. Never wear sandals or other open-toed shoes. Footwear must always cover the foot completely.
4. Never wear shorts or skirts in the lab.
5. When working with Bunsen burners or flames, lighted splints, matches etc., acrylic nails are not allowed.

Personal Protection safety rules

1. When working with equipment, hazardous materials, glassware, heat and chemicals, always wear safety glasses or googles or face shield.
2. Handling any toxic or hazardous agents, always wear the appropriate gloves.
3. When performing laboratory experiments, you must always wear a lab coat.
4. Before leaving the lab or eating, always wash your hands.
5. After experiment, you should always wash your hands with soap and water.
6. When using lab equipment and chemicals, be sure to keep your hands away from your body, mouth, eyes, face, and items you'll handle after removing your gloves (e.g., phone or laptop).

Chemical safety rules

1. Every chemical should be treated as dangerous and before you start an experiment, make sure you are fully aware of the hazards of the materials you'll be using.
2. Do not allow any solvent or chemicals to come into contact with your skin.
3. Never stack chemicals in the laboratory; containers must be stored upright, must not be stored in/on fume hoods, desks, or lab benches.
4. Do not place chemicals within two inches of the edge of a lab bench (to avoid accidental knocking off of bench).
5. Only materials you require for your work should be kept in your work area.
6. Before removing any of the contents from chemicals bottle, read the label twice.
7. Do not put unused chemicals back into their original container.
8. Flammable and volatile chemicals should only be used in a fume hood.
9. If a chemical spill occurs, clean it up right away.
10. Ensure that all chemical waste is disposed of properly.

Glassware and Sharps safety rules

1. Make sure that disposal containers for broken glass and sharps are well labelled and placed in low-traffic areas.

2. Properly dispose of broken glassware and sharps. If these items are contaminated with a hazardous substance, they need to be treated as hazardous waste and disposed in the appropriate waste container.
3. Never use cracked or chipped glassware; promptly discard these items in the broken glass container.
4. Do not stack beakers, flasks, etc.
5. Wear appropriate gloves to clean glassware; do not pile up dirty or clean glassware.
6. Wash glassware carefully (dirty water can hide glass fragments).

Electrical safety rules

1. Electrical equipment should be maintained by trained individuals only.
2. Never overload circuits; use surge protection power strips and ground fault circuit interrupters as needed.
3. Properly ground all electrical equipment.
4. Be sure that all cords are well placed, in good shape, and away from water. Keep cords out of aisles.
5. Immediately report any electrical failure or suspicious heating of equipment to the laboratory manager/supervisor.

References

https://www.labmanager.com/science-lab-safety-rules-guidelines-5727

Basic and general rules to be followed in the laboratory, NPPRL, by everyone who uses the facilities.

1. The laboratory must be kept neat and orderly at all times.
2. Keep the work area free from unnecessary apparatus, paper, chemicals, and waste.
3. Do not place chemicals within two inches of the edge of a lab bench (to avoid accidental knocking off of bench)
4. All spills must be cleaned up before continuing work or other tasks.
5. All paths to exits must be kept clear and unobstructed.
6. All paths to emergency shower and eyewash areas must remain unobstructed.
7. Rinse broken glassware before disposal into the “Glass Waste” container.
8. Safely transfer biohazardous waste into the proper waste container.
9. Transfer waste paper, gloves packing material and wood into the regular waste container.
10. Clean all your equipment and put it away before leaving.
11. Clean your work area completely before leaving.
12. Individuals using common equipment and facilities must clean up after use.
13. Return all unused chemicals to their proper storage places or on the laboratory cart.
14. Do not chew gum, drink, eat or apply lip balm or cosmetics while working in the lab.

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