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.