CROP PROTECTION PROGRAMME

Coconut lethal yellowing disease: development of new diagnostic tools and laboratory support to promote their application

R 8309 (ZA 0581)

FINAL TECHNICAL REPORT

ANNEX

Good practice in the molecular biology laboratory: diagnostic protocols

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Rothamsted Research

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GOOD PRACTICE IN THE MOLECULAR BIOLOGY LABORATORY

Coconut Programme

- Never put bags on work surfaces always on floor or in office
- Always wear a lab coat when in the lab, hang up when not in use NEVER wear it outside the laboratory.
- Keep work surfaces clean and tidy.
- Always wear gloves when working with chemicals especially those used to manipulate or stain DNA.

LABORATORY NOTE BOOK

- The lab notebook provides a means of recording all your experimental data, in such a way that it is verifiable by other people.
- Choose a notebook that has fixed pages.
- Number all pages consecutively in the top corner.
- On the first page place the following:
- Owner of the lab book (your name)
- Book Number
- Date lab book started
- Date lab book completed
- Names and signatures of those allowed to enter data into the lab book
- Number of pages in the lab book.

Record all your activities in the lab in the book on a daily basis. You should put the date at the start of each session and underline, sign and date at the completion of each session. Leave no gaps or blank pages.
Photographs etc can be stuck into the book to show results but say where the original data is – for example on a computer disk, hard drive or CD ROM.

Record all experimental data, results and observations in your laboratory notebook.
Protocols can be written out or typed and pasted into the rear of the book or a separate Protocol Laboratory Note Book. Protocols should be numbered so that they can be referred to without confusion. If you amend a protocol, note the details in your lab book and add the new protocol to the list.

DANGER ETHIDIUM BROMIDE

When working with this chemical try to contain it to a defined area. Do not move about the lab wearing gloves that are contaminated with EtBr. Dispose of contaminated gloves, gels, tips and tissue in a secure bag or box. When necessary dispose of this material by burning in an incinerator. Always wear gloves when working with EtBr.

Protocol No. 1
Collection of coconut palm inflorescence and trunk tissues for PCR detection of phytoplasmas

Choose trees with bark that is as clean as possible. If bark is covered with moss or lichen, remove with a knife before drilling.

Trunk wood

Each palm will have 3 holes drilled spaced as equally as possible around the trunk circumference and between 1m and 1,5m above ground level.

Clean and flame drill bit
Select drill sites, swab trunk surface with ethanol, flame if necessary
Drill through bark to remove outer layer, clean drill bit.
Drill hole to depth of 10cm collecting sawdust into clean polythene bag
Shake bag to mix well then divide sawdust into 2 parts:

1 for PCR directly into CTAB buffer
2 dry for ELISA.

Repeat steps for holes 2 and 3.

Samples should be labelled with the following information:
Number of palm - disease stage of palm – A, B or C for drill site.

Inflorescence

For palms at stages 1 – 3 of the disease it should be possible to collect intact unopened inflorescence tissue. The climber should cut the inflorescence without removing the spadix as close to the base as possible. Take one inflorescence per tree, from numbers 8, 9 or 10 as these will provide the highest concentration of phytoplasma DNA.
Record number of tree, number of inflorescence and disease stage.

Protocol No. 2

Phytoplasma Extraction from Coconut Palm Trunk Wood for PCR – CTAB method

See Protocol No. 1 for the collection of coconut palm trunk wood

It is important that samples are left in extraction buffer for at least 24h before continuing with this protocol.

1 Take 800ul of extract from the field collection tube
2 Transfer to sterile 2ml microfuge tube
3 Add equal volume of chloroform : Iso amyl alcohol (24:1) vortex
4 Centrifuge 4000rpm 10min
5 Carefully remove aqueous (upper) layer to clean tube
6 To aqueous layer add 800µl cold Iso propanol leave at least 2h or overnight at -20^{o} C
7 Centrifuge at 14,000rpm for 30min pour off supernatant
8 Wash pellet with 70% ethanol, **pour off carefully**
9 Dry pellet in vacuum desiccators (you may not see anything!)
10 Re-suspend pellet in 100µl TE
11 Store at 4C if using immediately or -20C for longer term

Reagents
CTAB (MIXED ALKYLTRI METHYL AMMONIUM BROMIDE (SIGMA M-7635)

CTAB BUFFER	to make 100ml solution	
CTAB	2%	2g
NaCl	1.4M	28ml of 5M solution
EDTA pH 8.0	20mM	4ml of 0.5M solution
Tris-HCl pH 8.0	100mM	10ml of 1M solution
PVP	1%	

PCR for phytoplasma detection

Two separate PCRs are done
PCR 1 - using primers P1 & P6 (or P7) – phytoplasma specific
PCR 2 - using primers G-813F & Awka SR (specific for CSPWD)

PRIMERS

Primer	Sequence 5'-3'	Reference
P1	*AAG AGT TTG ATC CTG GCT CAG GAT T*	Deng & Hiruki 1991
P6	*CGG TAG GGA TAC CTT GTT ACG ACT TA*	Deng and Hiruki 19
G-813F	*CTA AGT GTC GGG GGT TTC C*	

Method

Use Amersham-Pharmacia **Ready-To-Go™** PCR beads in a 25µl reaction mix containing 1µl of each primer (10pmol stock) and 1µl template DNA. Template DNAs from healthy or diseased palms are used as isolated. Positive DNA and water controls must e included in all PCR runs.

1. Number your PCR tubes note in lab book
2. Prepare the premix on cold box, compensate for any pipetting errors by including one extra volume, as follows:

Primer#1	1µl	x	no. of samples + 1
Primer#2	1µl	x	no. of samples + 1
SDW	21ul	x	no of samples + 1

e.g. If you have 12 samples, a positive and a water control you will need to multiply your volumes by 15.

3. Give short (10 sec) centrifugation to mix.
4. Pipette 23.0µl of this premix into each of your PCR tubes
5. Add 2.0µl of each template DNA to a separate tube
6. Add positive control DNA to one tube.
7. Add water=) control (1µl) to one tube.
8. Mix by short (10sec) centrifugation. Total volume per tube = 25.00µl.

Settings for the PCR machine

95^{o}C	3mins		1 cycle
94^{o}	30s	]	
53^{o}	1min30s		] 35 cycles
72^{0}	1min30s	]	
72^{0}	10mins		1 cycle

PCR products can be stored in the fridge at 4^{o}C or at -20C

Detecting the PCR products by agarose gel electrophoresis

Electrophorese through a 1% or 1.5% Agarose gel at 70volts using 1 x TBE buffer The gel contains Ethidium Bromide and visual examination and recording is done on the UV transilluminator. **Note:** We add ethidium bromide to our agarose gel and use 1% gels. This is a much better way than staining the gel in a large volume of ethidium bromide solution.

TBE buffer (5 X)

TRIS Base	54g
Boric Acid	27.5g
EDTA 0.5M pH8.0	20ml

make up to 1lt DH_2O

Ethidium bromide stock 1mg / ml

Add 1µl ethidium bromide stock per 20ml agar gel volume

Method

1. Dissolve agarose in TBE buffer by heating in the microwave until clear. Cool then add ethidium bromide.
2. Tape the ends of the frame and with the comb in place, carefully pour in the gel mix and allow to set.
3. Fill the tank with TBE buffer to cover the gel surface.
4. Loading volumes of product will vary, but the method of pipetting out 2µl of tracking dye onto film, adding the product and mixing, then directly loading saves time, tips and reserves any unused product for RFLP.
5. You may want to use a 1kb ladder at the start of each row **BUT remember to record the loading pattern.**

Protocol No. 3

Phytoplasma Extraction from Coconut Palm Trunk Wood for PCR – Qiagen DNeasy prep

See Protocol No. 1 for the collection of coconut inflorescence and trunk wood

This protocol can be used for both fresh and dried trunk samples and fresh inflorescence samples. Before starting you should make sure that you have numbered your tubes and have sufficient samples to process,
NB. ALWAYS process and EVEN number of samples so that the centrifuge will balance.

Preparation of the lysate mix

Fresh Inflorescence and wood samples

Remove the bottom 2cm of tissue from the cut inflorescence and discard. Take a slice of 1cm of the rachis tissue and chop finely, mix well and take 500mg (0.5 on balance) of tissue into mortar and add 400ul of buffer AP1 and 4ul RNase A stock, grind and transfer to fresh tube, Vortex and incubate at 65 C for 10-20 minutes. Mix 2-3 times during incubation by inverting tube.

Dried wood sample

Put 500ug (0.05 on balance) of wood into a microfuge tube add 400µl of buffer AP1 and 4 µl of RNase A stock solution. Mix with mini pestle or vortex then incubate at 65 C for 10 – 20 min. Mix 2-3 times during incubation by inverting tube.

The method the follows that given in the DNeasy instruction book but is essentially as follows:

1. Add 130ul of buffer AP2 to the lysate mix and incubate 5 min on ice
2. Apply lysate to the QiaShredder spin column (lilac) sitting on a 2 ml collection tube and centrifuges 2min at maximum speed.
3. Transfer the flow through fraction to a new tube without disturbing any pellet, discard collection tube.
4. Add 750ul buffer AP3/E to cleared lysate and mix by pipetting.
5. Apply 650ul of the mixture (including any precipitate which forms) to the DNeasy spin column sitting in a 2ml collection tube. Centrifuges for 1 min at 6000 g / 8000 rpm. Discard flow trough.
6. Repeat using remaining sample. Discard flow through and collection tube.
7. Place DNeasy column in a new collection tube, add 500ul buffer AW to the column and centrifuge for 2 min at 6000 g / 8000 rpm. Discard the flow through but reuse the collection tube.
8. Add 500ul buffer AW to the DNeasy column and centrifuge for 2 min at maximum speed. Discard flow through and collection tube.

9. Place DNeasy column on a new 1.5ml or 2ml microfuge tube and add 150ul of buffer AE –**preheated to 65C –** onto the DNeasy membrane. Incubate for 5 min at room temperature then centrifuge at 6000 g / 8000 rpm for 1 min.

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