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ASEAN DIAGNOSTIC PROTOCOLS FOR REGULATED PESTS *MICROCYCLUS ULEI* (SOUTH AMERICAN LEAF BLIGHT OF RUBBER)

ASEAN SECTORAL WORKING GROUP ON CROPS
2018

ASEAN Diagnostic Protocols for Regulated Pests

Microcyclus ulei (South American Leaf Blight of Rubber)

CONTENTS

Acknowledgement

This diagnostic protocol is made relevant with the majority of pest information from Food and Agriculture Organisation RAP Publication 2011/07 on Protection against South American Leaf Blight of rubber in Asia and the Pacific Region. The photos and morphological identification of SALB were contributed with kind courtesy by Michellin during the IV training workshop on SALB in Brazil from 13 to 17 Nov 2017. The detailed morphological keys of SALB were cited from "doenças da seringueira no Brasil" (1997) edited by the Embrapa.

The molecular assays were adopted from the paper 'Erasing the Past: A New Identity for the Damoclean Pathogen Causing South American leaf Blight of Rubber' by Hora Junior *et al.* 2014, which was further verified during the workshop in Brazil with the use of SALB pathogens. Additional information provided by the experts in the Michellin's IV training workshop on SALB in Brazil from 13 to 17 Nov 2017 has also been incorporated in this diagnostic protocol.

1. Pest Information

South American Leaf Blight (SALB) is the main invasive fungal pathogen of rubber trees caused by the ascomycete *Microcyclus ulei.* It inhibits natural rubber production which limit worldwide production of natural rubber. Till date, *M. ulei* is known to infect all original habitats of *Hevea* species but is still restricted to its continent of origin. However, it still may present a threat to South East Asia which accounts for 99% of the worldwide production of natural rubber.

Brazil, being the biological centre of origin of the rubber tree, accounts for the remaining 1% of world rubber production. This discrepancy is explained by the fact that rubber is produced on plantation in Asia, India, and Africa while Brazil relies on extractive production systems in the Amazon Basin for rubber. As the trees from the plantations in Central and South America are often destroyed by *M. ulei* before they reach physiological maturity, they have never reached full production of rubber thus far. It was also perhaps fortunate that SALB did not establish in South East Asia during the introduction period of rubber trees from South America.

The rapid spread of SALB causes severe leaf fall and twig dieback. Control measures such as breeding and selection as well as agronomical measures have been in placed but have yet been successful in circumventing the disease. Chemical controls were evaluated to be expensive and impractical due to the need for repeated application of fungicides to trees of great heights. Breeding and selection were found to be ineffective by the evolution of new physiological races of the pathogen that are capable of breaking down the resistance.

Young leaflets of up to 12 days old are most susceptible to infection by *M. ulei* and become increasingly resistant thereafter. Four to ten days after infection, lesions start appearing which produce conidia and lead to shredding of young diseased leaves. Leaflets with severe conidial infections shrivel, turn black and drop off. The most important stage of the disease is the increase in quantity of conidial inoculum which leads to physiological debilitation of the trees. Infection of older leaflets do not cause premature fall and remain on the tree. 30-60 days of post infection, black stromata develops followed by the asexual pycnidial phase and sexual ascospore stage of the pathogen. The disease cycle of SALB is as illustrated in Figure 1.

Ripe diseased leaves with stromata, provide ascospores as the primary source of inoculum which are readily dispersed by the wind. Once ascospores reach the young leaflets, they germinate, penetrate and colonize the tissue. Within 5 to 6 days, lesions covered with conidia on the infected leaflets may also be spread by wind or rain Thus, conidia are a secondary source of inoculum. Subsequently, formation of stromata takes two months which takes another one month for asci to form and one month for them to 'ripen' and to liberate the ascospores. The complete life cycle takes four or five months. The survival stage of the fungus is dependent on the stromatic ascogenous stage which persists on mature diseased leaves.

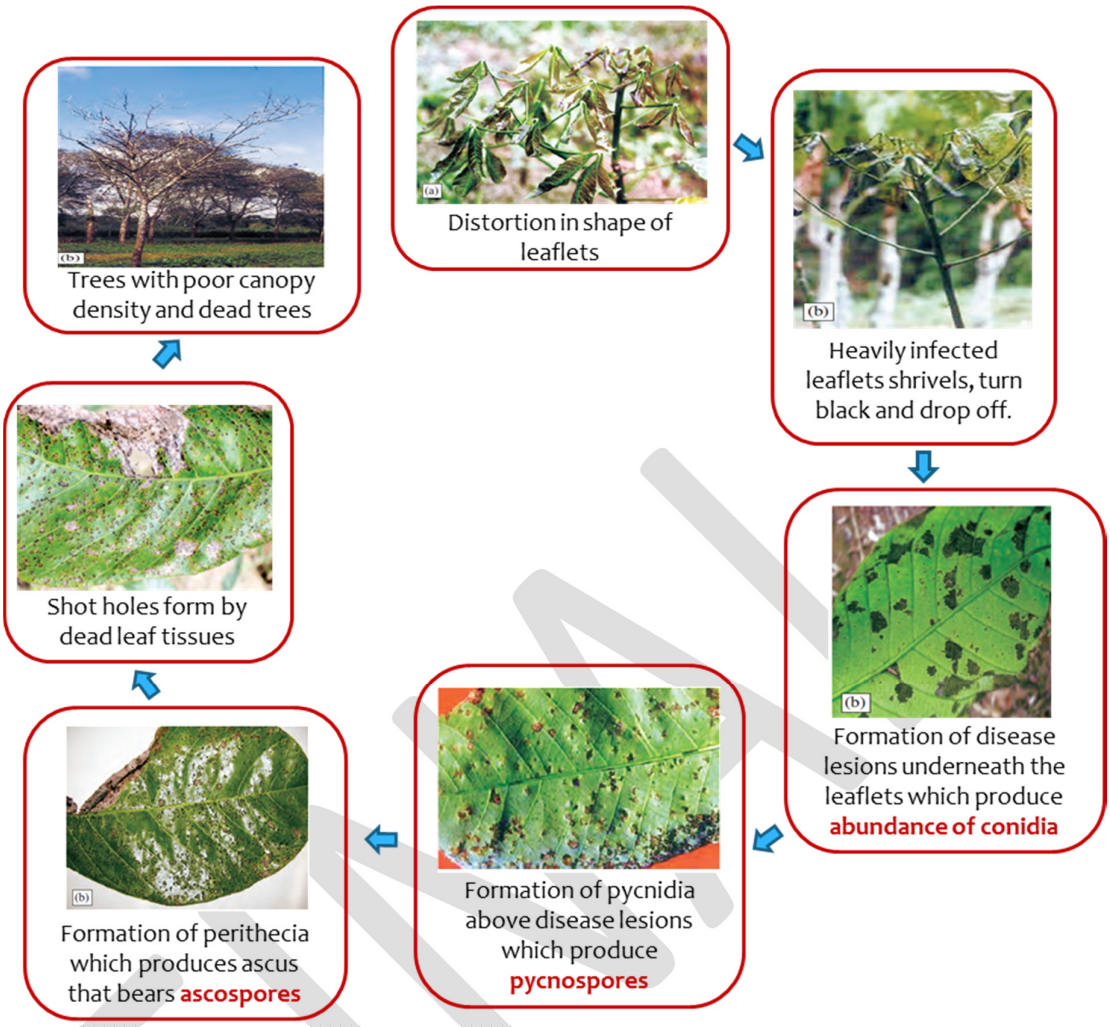

Fig.1. Disease cycle of SALB

2. Pest identity, taxonomy, hosts and plant parts affected

Pathogen:	*Microcyclus ulei* (P.Henn) v. Arx
Order:	Ascomycetes
Family:	Dothideales
Synonyms:	*Dothidella ulei* (Henn. 1904)
	Melanopsammopsis ulei (Henn.) Stahel 1917
	Aposphaeria ulei Henn. 1904
	(Conidial state: *Fusicladium macrosporum* Kuyper 1912)
	(*Pseudocerocospora ulei*)
Common name:	South American Leaf Blight (SALB)
Host species:	*Hevea brasiliensis* Muell. Arg. (Commercial species)
	Hevea benthamiana Muell. Arg.
	Hevea guianensis Aubl.
	Hevea spruceana (Benth.) Muell. Arg
Part of plants affected:	Young leaves severely affected. The young tissue of petioles, stems, inflorescences and fruit pods is less affected.

3. Detection & Identification

Diagnosis of *M.ulei* can be achieved by microscopic examination of the morphological characteristic of the mycelium grown on media and by molecular testing (Polymerase Chain Reaction- PCR). Positive and negative controls must be included for all tests (see section 3.4 for reference control).

3.1 Symptoms

The symptoms vary with the age of the infected leaves. In young leaves of up to 10 days old, slight discoloration and hypertrophic deformations appear three to four days after infection. Five to six days after infection, greyish to olive-green masses of conidia appear on the abaxial leaf surface (Fig.2A). Affected leaves will abscise if infection density is high. The petioles, young twigs and young fruits can also be affected (Fig.2B). Repeated defoliations and twig dieback weaken the tree which may eventually lead to the death of young trees.

In young leaves that are older than 12- 15 days, the lesions become smaller and slightly hypertrophic. Conidiospore production is low or even absent. In slightly infected young leaves or infected old leaves, black stromatic areas form on the ataxia leaf surface without abscising

known as the pycnidia (Fig 2C). The stromata contain pycnidial cavities in which conidia are formed. Subsequently, the stromatic areas coalesce to form ring-like structures (Fig. 2C and 3). The leaf tissue within the ring disintegrates resulting in small holes.

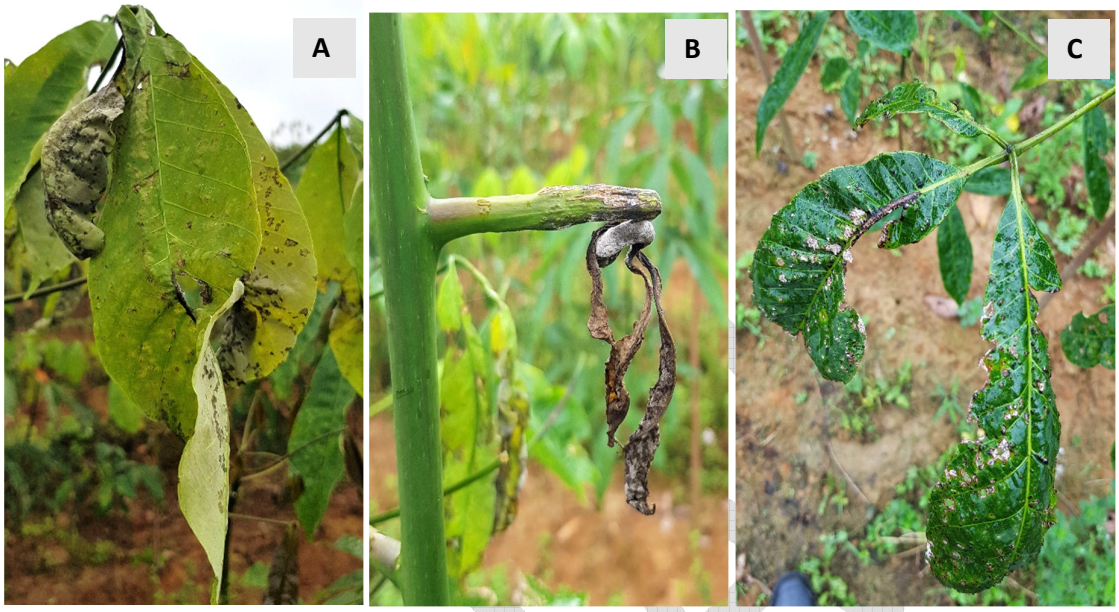

Fig 2. Symptoms of SALB infection on different tree parts and disease cycle. **A:** Greyish masses of conidia on abaxial leaf surface resulting in deformation. **B**: Petioles affected by SALB. **C:** Black stromatic areas formed on the ataxia leaf surface which eventually result in shot holes.

Fig. 3. Stromatic areas coalesce to form ring-like structure

3.2 Isolation and culturing of *M.ulei*

M.ulei can be isolated and cultured on Potato Sucrose/ Dextrose Agar (PSA/PDA). Also, the operative method preparation of the culture medium for *M.ulei* was also described by Saulo Cardoso and Carlos Mattos (Refer to Appendix 1). Disease lesions are excised with a sterile scalpel, surface disinfected with 2 % sodium hypochlorite (NaOCl) for 2 min, rinsed twice in sterile distilled water and blotted dry. Subsequently, the lesions are placed aseptically on Petri

dishes (9cm in diameter) on PSA or PDA. The growth of the fungus is very slow and forms stoma either raised above the surface of the medium or flattened along the media surface (Fig.4). Conidia are produced on artificial medium especially on special medium for spore production. Exposure to intermittent light and dark periods effectively enhanced conidal production.

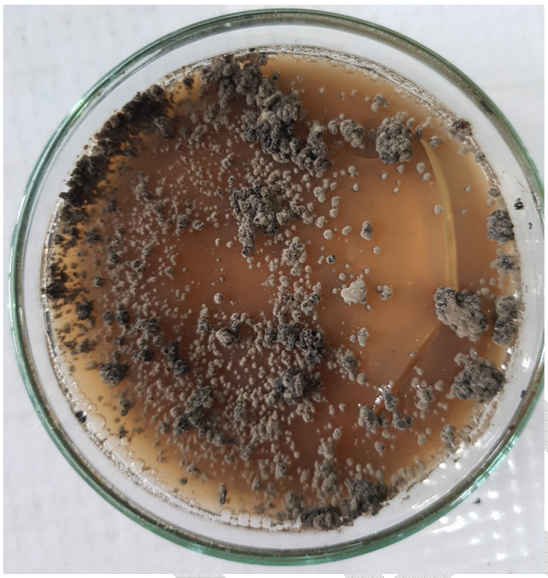

Fig 4. Growth of SALB stroma above the media surface

3.3 Morphological Characteristics

M. ulei produces three types of spores; conidia on immature leaves; pycnospores on newly matured leaves; and ascospores on fully matured leaves. The main propogules are conidia and ascospores. Pycnospores do not appear to germinate and do not cause infection unlike conidia and ascospores.

The first conidial phase of *M.ulei* is expressed in the structure of simple, erect or geniculate conidiophores with one to four conidial scars. The conidiophores are slightly greenish and formed from the subepidermal stromal tissue, measuring up to 140 x 4-7um. Conidia have polyblastic origin, are hyaline to light brown, of sui generis formats, ranging from straight to sinuous or in the form of a corkscrew or peanut pod, with smooth outer walls or slightly verrucous walls, truncated bases, uni or bicellular (rarely tricellular). The unidirectional conidia measure 15-43 x 5-9um and the bicellular, 23-63 x 5-10um (Ellis, 1976; Holliday, 1980) (Fig 5A).

The second asexual or anamorphic-picnidial phase is present in black (exoepidermal), carbonaceous, stromes, which are rounded or grouped in mounds, especially in the upper face of the leaflets. the pycnidia are ostiolate, measure 120-160um in diameter, have simple or branched conidiophores arising from hyphae that make up the picnidial wall and produce conidia in a phialidico-enteroblastic manner (Sutton, 1980); cylindrical conidia with dilated ends (resembling human femurs), 12-20 x 2-3um (Fig.5B).

The telomorphic or sexuated phase of *M.ulei* are produced in ascostromes or pseudothecios within the black exoepidermal stroma. They are usually about 200-500um in diameter, laterally aggregated, forming circles or mounds, especially on the upper surface of the foliolos. These stroma are black, carbonaceous, and have rough outer walls. The ascos are bitunicados, nailed, 50-80 x 12-16um, and they carry eight ascospores; these are hyaline, irregularly ellipsoidal to fusoid, bicellular, with smooth construction in the septa dividing the ascospores into two unequal cells; the major, with a sharper portion, and oriented to the base of disgust. The ascospores measure 12-20 x 2-5um (Fig.5C).

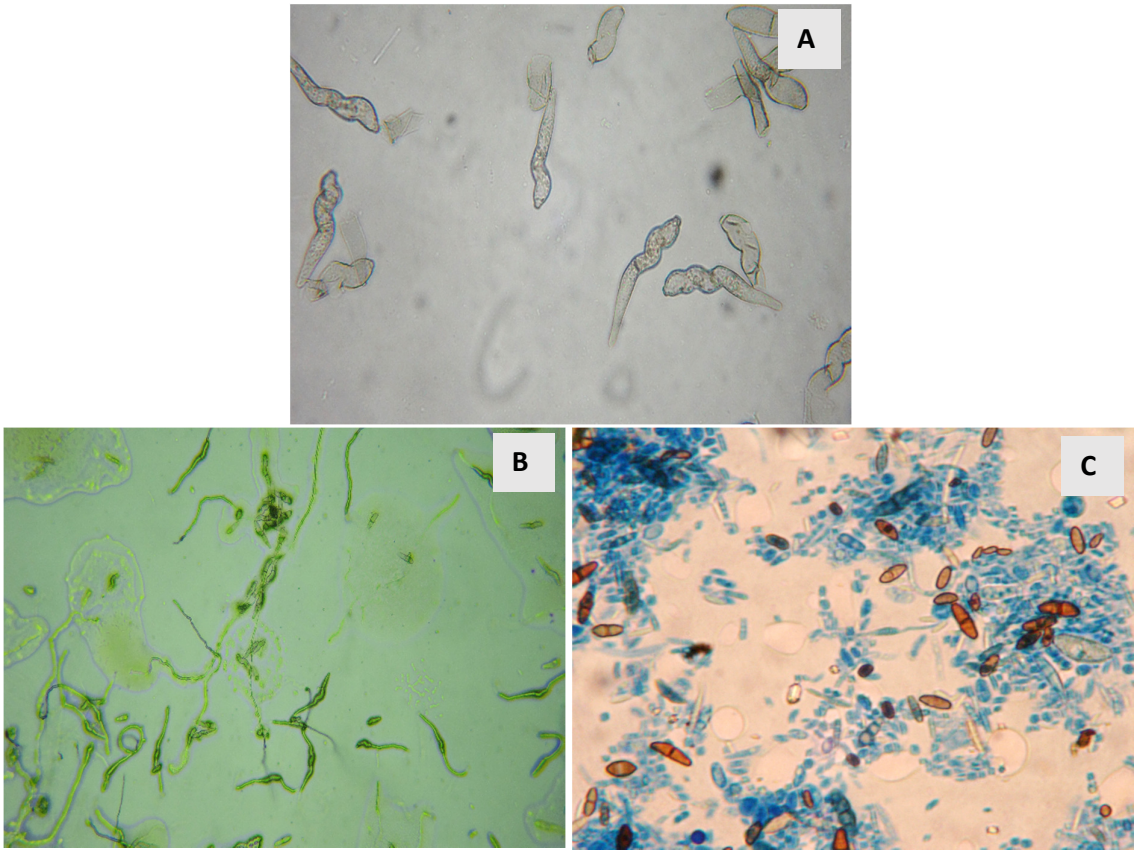

Fig. 5 Three different stages and spores produce by *M.ulei.*
A: Conidiospores. **B:** Pycnospores. **C:** Ascospores

3.4 Molecular detection

3.4.1. Controls for molecular testing

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – are essential. For PCR, a positive nucleic acid control, an internal control and a negative amplification control (no template

control) are the minimum controls that should be used. These and other controls that should be considered for each series of nucleic acid extractions from your test samples as described below.

Positive nucleic acid control. Pre-prepared (stored) nucleic acid, whole genome DNA or a synthetic control (e.g. a cloned PCR product) may be used as a control to monitor the efficiency of PCR amplification. Refer to Appendix 3 for synthesized gene sequence of SALB.

Internal controls. For conventional PCR, a fungal housekeeping gene (HKG) such as COX (Weller *et al.,* 2000), 16S ribosomal (r)DNA (Weisberg *et al.,* 1991) or GAPDH (Mafra *et al.,* 2012) should be incorporated into the PCR protocol as a control to eliminate the possibility of false negatives due to nucleic acid extraction failure or degradation of the presence of PCR inhibitors.

Negative amplification control (no template control). For conventional and real-time PCR, PCR- grade water that was used to prepare the reaction mixture is added at the amplification stage to rule out false positives due to contamination during preparation of the reaction mixture.

Positive extraction control. This control is used to ensure that nucleic acid from the target is of sufficient quality for PCR amplification. Nucleic acid is extracted from infected host tissue or healthy plant tissue that has been spiked with the target at the concentration considered the detection limit of the protocol.

The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the DNA extraction. For PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples. If required, the positive control used in the laboratory should be sequenced so that the sequence can be readily compared with sequences obtained from PCR amplicons of the correct size. Alternatively, synthetic positive controls can be made with a known sequence, which, again, can be compared to PCR amplicons of the correct size.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and cross-reaction with the host tissue. The control comprises of nucleic acid that is extracted from uninfected host tissue and subsequently amplified. Multiple controls are recommended when large numbers of positive samples are tested.

3.4.2 DNA extraction

DNA can been successfully extracted from lesions or plant material using GeneAll Exgene Plant SV kit according to the manufacturer's protocol (GeneAll) or Cetyl trimethylammonium bromide (Refer to Appendix 2). Prior to DNA extraction, pycnidial stromata of *A.* ulei or ascostromata of *M. ulei* are excised from a single lesion of an infected leaf with a sterilized razor blade. Each lesion is examined under the microscope to check for possible contamination by mycoparasites and selected stromata (approximately 10 structures) are transferred to a microtube (1.5mL). The procedure is repeated from another lesion on the same leaf. To break up the

melanised cell walls, the microtubes containing fungal material (mycelium, pycnidia or ascostromata) are placed in liquid nitrogen and macerated using a micropestle.

3.4.3 Conventional PCR

Several primer pairs are available for diagnosis of *M. ulei*. Hora Junior *et al.* (2014) primers LROR and LR5 target 28s rRNA gene (LSU), ITS1 and ITS4 target the first and second internal transcribed spacer (ITS), NMS1 and NMS2 target the mitochrondrial region of the mtSSU-rDNA, Mcm7-709for and Mcm7-1384rev target the partial sequences of nuclear genes such as the mini-chromosome maintenance protein (MCM7), EF1-728F and EF1-986R target the translation elongation factor 1-alpha (EF-1α), and ACT-512F and ACT-783R target the actin gene respectively. These primers identify strains including *Microcyclus ulei*, *Flusicladium heveae* and *Aposphaeria ulei*.

PCR protocol of Hora Junior *et al.* (2014)

The primers are:

ITS1 (Forward): 5'- TCCGTAGGTGAACCTGCGG- 3'
ITS4 (Reverse): 5' – TCCTCCGCTTATTGATATGC- 3'

LROR (Forward): 5' – ACCCGCTGAACTTAAGC- 3'
LR5 (Reverse): 5'- ACCCGCTGAACTTAAGC- 3'

NMS1 (Forward): 5'- CAGCAGTGAGGAATATTGGTCAATG-3'
NMS2 (Reverse): 5'- GCGGATCATCGAATTAAATAACAT-3'

Mcm7-709 (Forward): 5'- ACIMGIGTITCVGAYGTHAARCC-3'
Mcm7-1384 (Reverse): 5'- GAYTTDGCIACICCIGGRTCWCCCAT-3'

EF1-728 (Forward): 5'- CATCGAGAAGTTCGAGAAGG-3'
EF1-986 (Reverse): 5'- TACTTGAAGGAACCCTTACC-3'

ACT-512F (Forward): 5'-ATGTGCAAGGCCGGTTTCGC-3'
ACT- 783R (Reverse): 5'- TACGAGTCCTTCTGGCCCAT-3'

The Polymerase Chain Reaction (PCR) mixture is prepared in a sterile tube and consists of 5X MyTaq Buffer, 10pmol/µL of each primer and 5u/µL of Taq DNA polymerase as described by the manufacturer (Bioline). Extracted DNA sample volume of 3 µL is added to 47 µL of the PCR mixture to give a total of 50 µL per reaction. The reaction conditions are an initial denaturation step of 94˚C for 5 min, followed by 35 cycles of 30s at 94 ˚C, 30s at 60 ˚C and 1 min at 72 ˚C, with a final extension of 10 min at 72 ˚C.

3.4.4 Interpretation of results from conventional PCR

The pathogen-specific PCR will be considered valid only if the below criteria are met:
- The positive control produces the correct size amplicon for the fungi i.e 600bp for ITS & 900bp for LSU.
- No amplicons of the correct size for the fungi are produced in the negative extraction control and the negative amplification control

If 18S rDNA internal control primers are also used, then the negative (healthy plant tissue) control (if used), positive control, and each of the test samples will produce an approximately 1.6 kilobase (kb) band (amplicon size will depend on which 16s rDNA primers are used. Note that synthetic and plasmid positive controls will not produce a 1.6 kb band. Failure of the samples to amplify with the internal control primers suggests, for example, that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract, or the DNA has degraded.

- A sample will be considered positive if it produces an amplicon of the correct size of 600bp for ITS & 900bp for LSU.

3.5 Identification

Identification of presumptive *Microcyclus ulei* should be verified by two or more techniques. In addition to observing morphological characteristics on media, molecular testing should be performed for confirmation.

3.5.1 PCR methods

It is recommended that in addition to the PCR protocol described in section 3.4.3, the identification of pure cultures of suspect strains is confirmed by using two different sets of primers. This is because of the findings that currently, most published primer pairs lack specificity. The primer pairs of Hora Junior *et al.* (2014) have been found to be specific to extracted DNA from pure cultures but not so for extracted DNAs from suspected infested leaf tissues.

Identification can be further confirmed by sequencing the resulting PCR amplicons and comparing their sequences with those of *Microcyclus ulei* deposited in the European Bioinformatics Institute (EMBL-EBI) GenBank database.

3.5.2 Sequence analysis

PCR products should be sequenced either directly or by first cloning them into a PCR cloning vector. Sequence data can be analysed using the Basic Local Alignment Search Tool, BLAST, available at the European Bioinformatics Institute (EMBL-EBI) GenBank (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html). If the sequence shares less than 97.5%

identity with its closest relative, the *Microcyclus* is considered to be a new '*Microcyclus ulei'* species. Sequencing a separate region of the genome such as the mitochondrial region of the mtSSU-rDNA, mini-chromosome maintenance protein (MCM7), translation elongation factor 1-alpha (EF-1α) and actin gene is also desirable.

3.6 Records

Records and evidence should be retained as described in ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance and where the *Microcyclus* is found in an area for the first time, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability:

- The original sample, kept frozen at -80˚C, or freeze-dried or dried over calcium chloride and kept at 4˚C.
- If relevant, DNA extractions should be kept at -20˚C or at -80˚C. Plant extracts spotted on membranes should be kept at room temperature.
- If relevant, PCR amplification products should be kept at -20˚C or at -80˚C.

4 References

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Appendix

Appendix 1. Operative Method for preparation of the culture medium for *M.ulei*

Include the following components to prepare 1 litre of culture medium for the isolation and growth of mycelium for *M.ulei*

- Boil 250grams of peeled potato in 700ml of distilled water
- Prepare 200ml of distilled water of the following product
  - 10grams of Sucrose
  - 2grams of Monobasic Potassium Phosphate
  - 0.15grams of Chloramphenicol
- Pass the potato broth through a fine sieve after cooking
- Cool the potato broth and add the reagents as mentioned in Step 2
- Add the following amino acids in a laminar flow hood
  - 1ml of lysine hydrochloride at a concentration of 10,000ppm
  - 0.1ml of Tryptophan at the concentration of 2,500ppm
  - 0.1ml of Threonine at a concentration of 2,500ppm
- Adjust the solution to pH 5.0± 0.2 with 1N of HCl or NaOH
- Add 20grams of Agar and dissolve in the microwave oven
- Transfer the medium to a 1000ml beaker and make up to volume with distilled water
- Transfer the hot medium to test tubes and autoclave at 121˚C at 15mins
- Tilt the tubes to solidify and store in the refrigerator after autoclaving

Appendix 2. High quality genomic DNA extraction using CTAB

- Prepare the following buffers prior to extraction of DNA
  - Buffer A: 0.35 M Sorbitol, 0.1 M Tris-HCl pH 9 and 5mM EDTA pH 8
  - Buffer B: 0.2 M Tris-HCl pH 9, 50mM EDTA pH 8, 2M NaCl and 2% CTAB
  - Buffer C: 5% Sarkosyl (N-lauroylsarcosine sodium salt Sigma L5125)
  - Lysis buffer for 17.5ml: 2.5 vol of Buffer A, 2.5 vol of Buffer B, 1.0 vol of Buffer C, 0.1% PVP and Proteinase K.
- Pre-warm Buffer B at 65 ˚C
- Prepare the lysis buffer using 17.5ml per 500mg of starting material
- Grind tissue using liquid nitrogen in a mortar, and transfer 500mg of finely ground powder in a 50ml Falcon tube

- Add 17.5ml of lysis buffer to the tube and mix by vortexing
- Incubate for 30min at 65 ˚C and mix frequently by inverting the tube
- Add 5.75ml (0.33vol) of KAc (5M), mix by inverting the tube and incubate on ice for 30mins
- Centrifuge for 20min at 4,000g at 4 ˚C
- Transfer the supernatant in a new 50ml Falcon tube and add 1 vol of Chloroform: Isoamyl alcohol (24:1)
- Centrifuge for 10min at 4,000g at 4 ˚C
- Transfer the aqueous phase in a 50ml centrifuge tube
- Add 100µl of RNAse A (10mg/ml) and incubate for 90- 120min at 37 ˚C
- Add 1/10 vol of NaAc and 1 vol of isopropanol (room temperature) and incubate for 5min at room temperature
- Centrifuge for 30min at 10,000g at 4 ˚C
- Discard the supernatant
- Wash the pellet with 2ml of ethanol (70%) and centrifuge for 10 min at 10,000g at 4 ˚C
- Discard the supernatant and dry the pellet for 5min at room temperature
- Resuspend the pellet in 500µl of TE at 65 ˚C and store at -80 ˚C

Appendix 3: Synthesized SALB gene (ITS and 28s rRNA)

GTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACCGAGCGAGGGCCGCCGCCC
GACCTCCAACCCCTCATGTGAACCCCACCCGTTGCTTCGGGGGCGACCCCGCCGGCACGCGTGCCGGCGCC
GGGCGCCCCCGGAGGTCTCCTCAACACGGCATCTTCTGCGTCGGAGCGTCACAGCAAATGACACAAAACTTT
CAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG
AATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTCGAG
CGTCATTACACCCCTCAAGCCCCGGCTTGGTGTTGGGCGTCGCGGCCCCGCGCGCCTCAAAGTCTCCAGCCA
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GGGATGAACCGAACGCGAGGTTAAGGTGCCGGAATGTACGCTCATCAGACACCACAAAAGGTGTTAGTTCA

TCTAGACAGCAGGACGGTGGCCATGGAAGTCGGAATCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAAT
GAACTAGCCCTGAAAATGGATGGCGCTCAAGCGTACTACCCATACCTCGCCGCCAGGGTAGAAACGATGCC
CTGGCGAGTAGGCAGGCGTGGGGGCTCGTGACGAAGCCTTCGGAGTGATCCGGGGTCGAACAGCCTCTAG
TGAGAC

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South American Leaf Blight (SALB) is the main invasive fungal pathogen of rubber trees caused by the ascomycete *Microcyclus ulei.* It inhibits natural rubber production which limit worldwide production of natural rubber. Till date, *M. ulei* is known to infect all original habitats of *Hevea* species but is still restricted to its continent of origin. However, it still may present a threat to South East Asia which accounts for 99% of the worldwide production of natural rubber.

Brazil, being the biological centre of origin of the rubber tree, accounts for the remaining 1% of world rubber production. This discrepancy is explained by the fact that rubber is produced on plantation in Asia, India, and Africa while Brazil relies on extractive production systems in the Amazon Basin for rubber. As the trees from the plantations in Central and South America are often destroyed by *M. ulei* before they reach physiological maturity, they have never reached full production of rubber thus far. It was also perhaps fortunate that SALB did not establish in South East Asia during the introduction period of rubber trees from South America.

The rapid spread of SALB causes severe leaf fall and twig dieback. Control measures such as breeding and selection as well as agronomical measures have been in placed but have yet been successful in circumventing the disease. Chemical controls were evaluated to be expensive and impractical due to the need for repeated application of fungicides to trees of great heights. Breeding and selection were found to be ineffective by the evolution of new physiological races of the pathogen that are capable of breaking down the resistance.

Young leaflets of up to 12 days old are most susceptible to infection by *M. ulei* and become increasingly resistant thereafter. Four to ten days after infection, lesions start appearing which produce conidia and lead to shredding of young diseased leaves. Leaflets with severe conidial infections shrivel, turn black and drop off. The most important stage of the disease is the increase in quantity of conidial inoculum which leads to physiological debilitation of the trees. Infection of older leaflets do not cause premature fall and remain on the tree. 30-60 days of post infection, black stromata develops followed by the asexual pycnidial phase and sexual ascospore stage of the pathogen. The disease cycle of SALB is as illustrated in Figure 1.

Ripe diseased leaves with stromata, provide ascospores as the primary source of inoculum which are readily dispersed by the wind. Once ascospores reach the young leaflets, they germinate, penetrate and colonize the tissue. Within 5 to 6 days, lesions covered with conidia on the infected leaflets may also be spread by wind or rain Thus, conidia are a secondary source of inoculum. Subsequently, formation of stromata takes two months which takes another one month for asci to form and one month for them to 'ripen' and to liberate the ascospores. The complete life cycle takes four or five months. The survival stage of the fungus is dependent on the stromatic ascogenous stage which persists on mature diseased leaves.

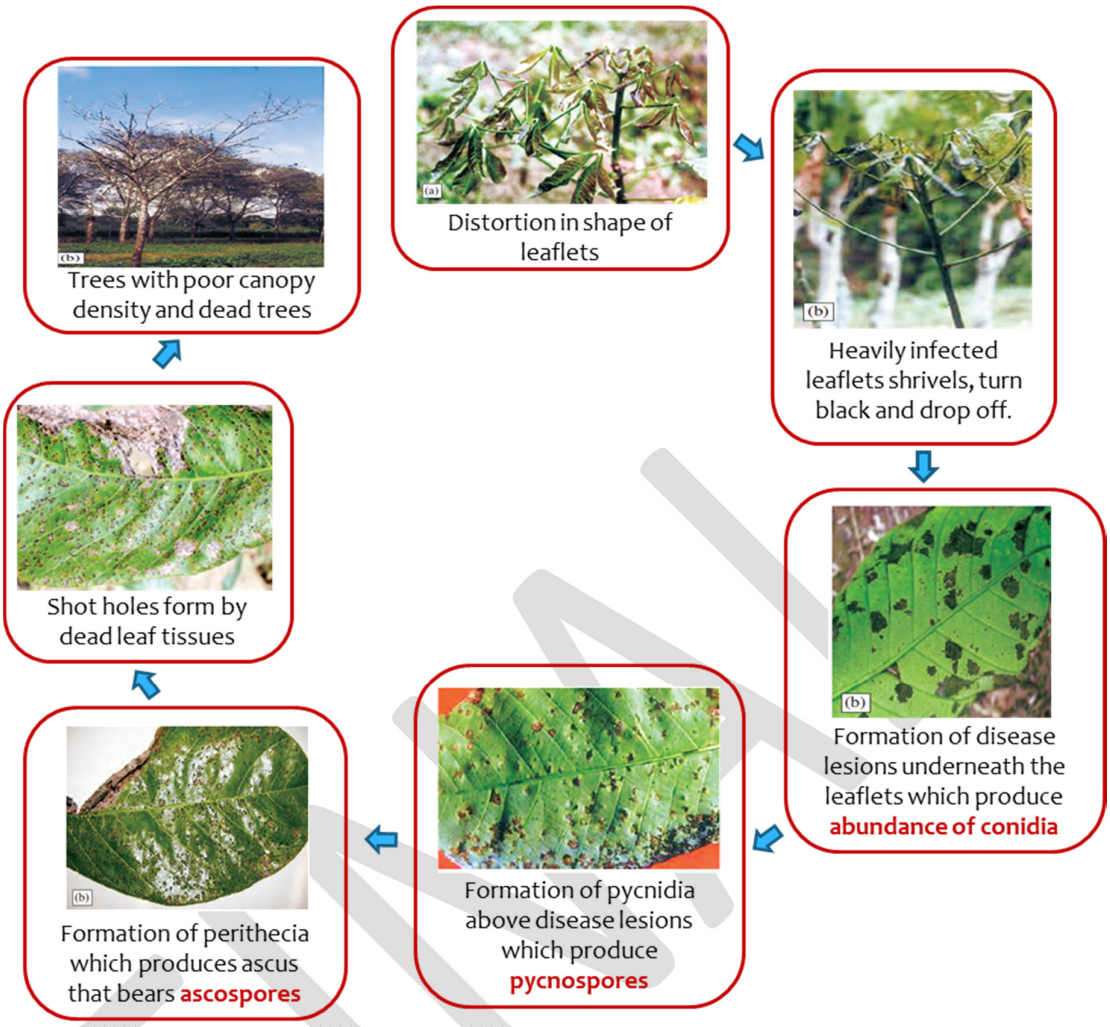

Fig.1. Disease cycle of SALB

2. Pest identity, taxonomy, hosts and plant parts affected

Pathogen:	*Microcyclus ulei* (P.Henn) v. Arx
Order:	Ascomycetes
Family:	Dothideales
Synonyms:	*Dothidella ulei* (Henn. 1904)
	Melanopsammopsis ulei (Henn.) Stahel 1917
	Aposphaeria ulei Henn. 1904
	(Conidial state: *Fusicladium macrosporum* Kuyper 1912)
	(*Pseudocerocospora ulei*)
Common name:	South American Leaf Blight (SALB)
Host species:	*Hevea brasiliensis* Muell. Arg. (Commercial species)
	Hevea benthamiana Muell. Arg.
	Hevea guianensis Aubl.
	Hevea spruceana (Benth.) Muell. Arg
Part of plants affected:	Young leaves severely affected. The young tissue of petioles, stems, inflorescences and fruit pods is less affected.

3. Detection & Identification

Diagnosis of *M.ulei* can be achieved by microscopic examination of the morphological characteristic of the mycelium grown on media and by molecular testing (Polymerase Chain Reaction- PCR). Positive and negative controls must be included for all tests (see section 3.4 for reference control).

3.1 Symptoms

The symptoms vary with the age of the infected leaves. In young leaves of up to 10 days old, slight discoloration and hypertrophic deformations appear three to four days after infection. Five to six days after infection, greyish to olive-green masses of conidia appear on the abaxial leaf surface (Fig.2A). Affected leaves will abscise if infection density is high. The petioles, young twigs and young fruits can also be affected (Fig.2B). Repeated defoliations and twig dieback weaken the tree which may eventually lead to the death of young trees.

In young leaves that are older than 12- 15 days, the lesions become smaller and slightly hypertrophic. Conidiospore production is low or even absent. In slightly infected young leaves or infected old leaves, black stromatic areas form on the ataxia leaf surface without abscising

known as the pycnidia (Fig 2C). The stromata contain pycnidial cavities in which conidia are formed. Subsequently, the stromatic areas coalesce to form ring-like structures (Fig. 2C and 3). The leaf tissue within the ring disintegrates resulting in small holes.

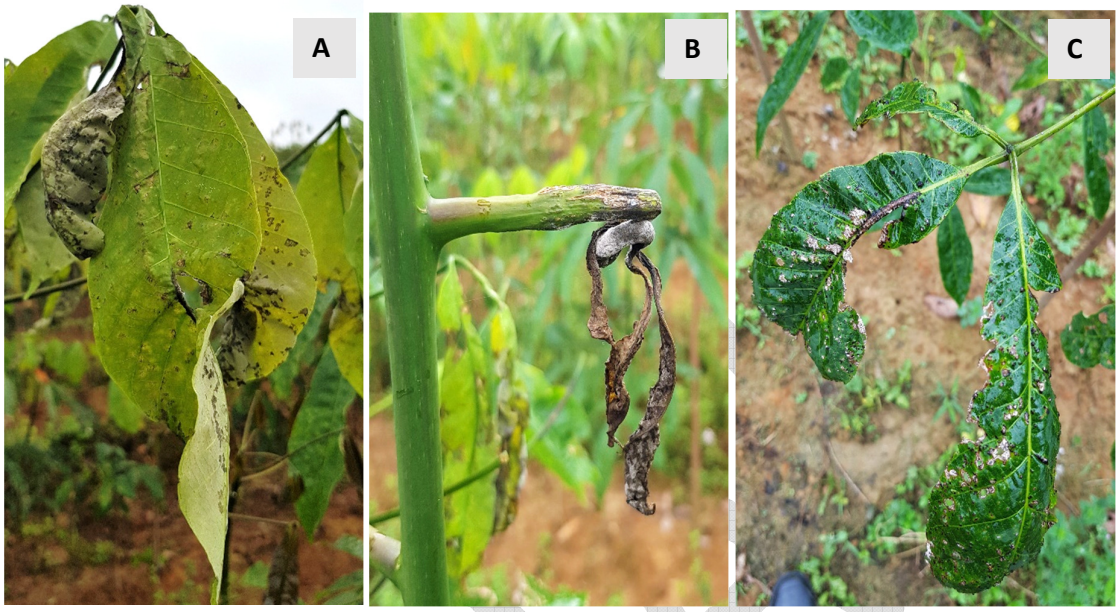

Fig 2. Symptoms of SALB infection on different tree parts and disease cycle. **A:** Greyish masses of conidia on abaxial leaf surface resulting in deformation. **B**: Petioles affected by SALB. **C:** Black stromatic areas formed on the ataxia leaf surface which eventually result in shot holes.

Fig. 3. Stromatic areas coalesce to form ring-like structure

3.2 Isolation and culturing of *M.ulei*

M.ulei can be isolated and cultured on Potato Sucrose/ Dextrose Agar (PSA/PDA). Also, the operative method preparation of the culture medium for *M.ulei* was also described by Saulo Cardoso and Carlos Mattos (Refer to Appendix 1). Disease lesions are excised with a sterile scalpel, surface disinfected with 2 % sodium hypochlorite (NaOCl) for 2 min, rinsed twice in sterile distilled water and blotted dry. Subsequently, the lesions are placed aseptically on Petri

dishes (9cm in diameter) on PSA or PDA. The growth of the fungus is very slow and forms stoma either raised above the surface of the medium or flattened along the media surface (Fig.4). Conidia are produced on artificial medium especially on special medium for spore production. Exposure to intermittent light and dark periods effectively enhanced conidal production.

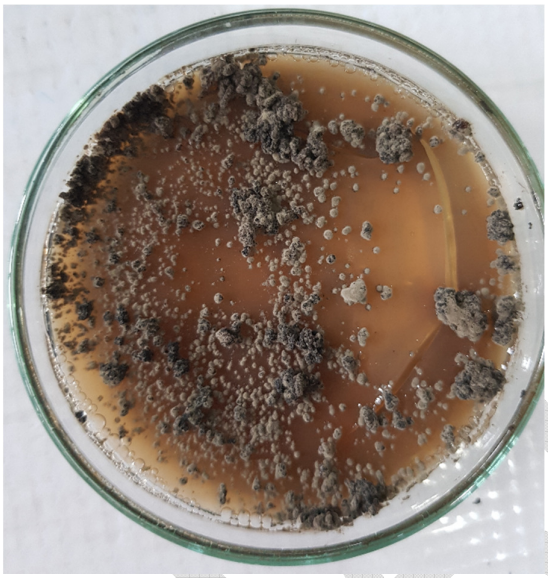

Fig 4. Growth of SALB stroma above the media surface

3.3 Morphological Characteristics

M. ulei produces three types of spores; conidia on immature leaves; pycnospores on newly matured leaves; and ascospores on fully matured leaves. The main propogules are conidia and ascospores. Pycnospores do not appear to germinate and do not cause infection unlike conidia and ascospores.

The first conidial phase of *M.ulei* is expressed in the structure of simple, erect or geniculate conidiophores with one to four conidial scars. The conidiophores are slightly greenish and formed from the subepidermal stromal tissue, measuring up to 140 x 4-7um. Conidia have polyblastic origin, are hyaline to light brown, of sui generis formats, ranging from straight to sinuous or in the form of a corkscrew or peanut pod, with smooth outer walls or slightly verrucous walls, truncated bases, uni or bicellular (rarely tricellular). The unidirectional conidia measure 15-43 x 5-9um and the bicellular, 23-63 x 5-10um (Ellis, 1976; Holliday, 1980) (Fig 5A).

The second asexual or anamorphic-picnidial phase is present in black (exoepidermal), carbonaceous, stromes, which are rounded or grouped in mounds, especially in the upper face of the leaflets. the pycnidia are ostiolate, measure 120-160um in diameter, have simple or branched conidiophores arising from hyphae that make up the picnidial wall and produce conidia in a phialidico-enteroblastic manner (Sutton, 1980); cylindrical conidia with dilated ends (resembling human femurs), 12-20 x 2-3um (Fig.5B).

The telomorphic or sexuated phase of *M.ulei* are produced in ascostromes or pseudothecios within the black exoepidermal stroma. They are usually about 200-500um in diameter, laterally aggregated, forming circles or mounds, especially on the upper surface of the foliolos. These stroma are black, carbonaceous, and have rough outer walls. The ascos are bitunicados, nailed, 50-80 x 12-16um, and they carry eight ascospores; these are hyaline, irregularly ellipsoidal to fusoid, bicellular, with smooth construction in the septa dividing the ascospores into two unequal cells; the major, with a sharper portion, and oriented to the base of disgust. The ascospores measure 12-20 x 2-5um (Fig.5C).

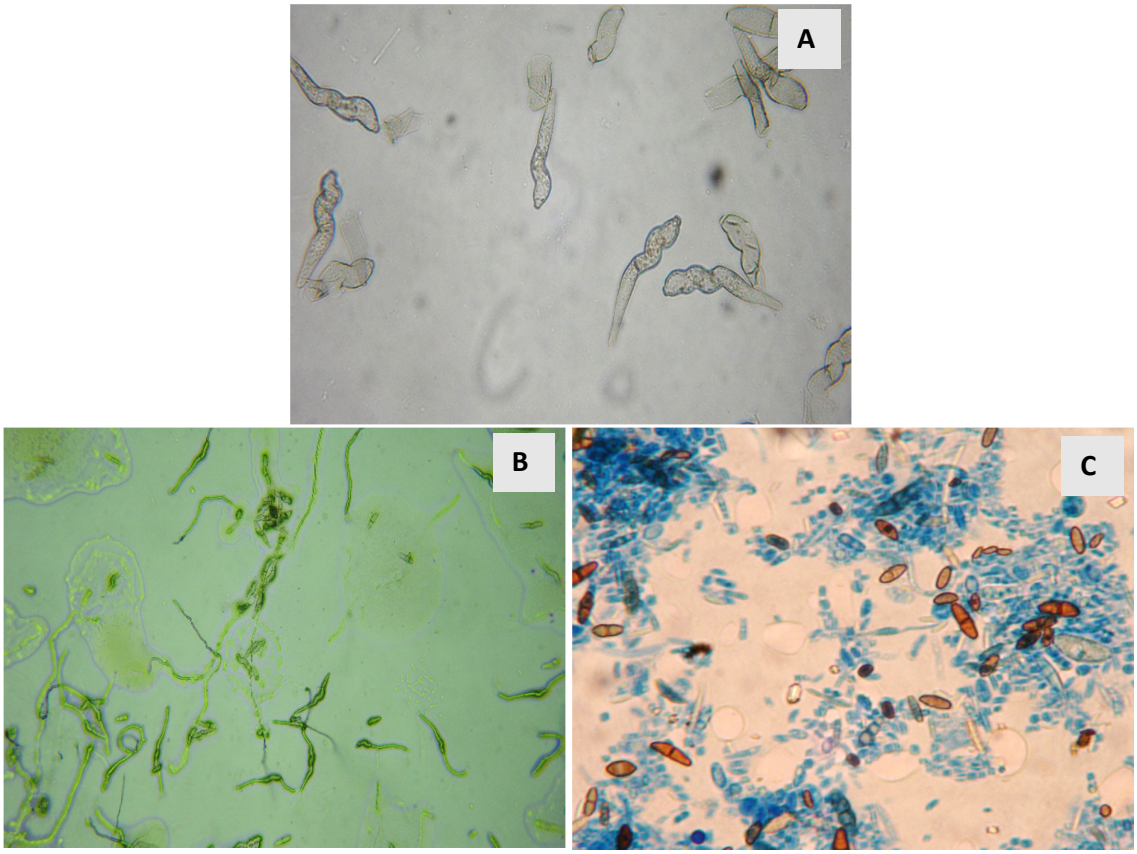

Fig. 5 Three different stages and spores produce by *M.ulei.*
A: Conidiospores. **B:** Pycnospores. **C:** Ascospores

3.4 Molecular detection

3.4.1. Controls for molecular testing

For the test result obtained to be considered reliable, appropriate controls – which will depend on the type of test used and the level of certainty required – are essential. For PCR, a positive nucleic acid control, an internal control and a negative amplification control (no template

control) are the minimum controls that should be used. These and other controls that should be considered for each series of nucleic acid extractions from your test samples as described below.

Positive nucleic acid control. Pre-prepared (stored) nucleic acid, whole genome DNA or a synthetic control (e.g. a cloned PCR product) may be used as a control to monitor the efficiency of PCR amplification. Refer to Appendix 3 for synthesized gene sequence of SALB.

Internal controls. For conventional PCR, a fungal housekeeping gene (HKG) such as COX (Weller *et al.,* 2000), 16S ribosomal (r)DNA (Weisberg *et al.,* 1991) or GAPDH (Mafra *et al.,* 2012) should be incorporated into the PCR protocol as a control to eliminate the possibility of false negatives due to nucleic acid extraction failure or degradation of the presence of PCR inhibitors.

Negative amplification control (no template control). For conventional and real-time PCR, PCR- grade water that was used to prepare the reaction mixture is added at the amplification stage to rule out false positives due to contamination during preparation of the reaction mixture.

Positive extraction control. This control is used to ensure that nucleic acid from the target is of sufficient quality for PCR amplification. Nucleic acid is extracted from infected host tissue or healthy plant tissue that has been spiked with the target at the concentration considered the detection limit of the protocol.

The positive control should be approximately one-tenth of the amount of leaf tissue used per plant for the DNA extraction. For PCR, care needs to be taken to avoid cross-contamination due to aerosols from the positive control or from positive samples. If required, the positive control used in the laboratory should be sequenced so that the sequence can be readily compared with sequences obtained from PCR amplicons of the correct size. Alternatively, synthetic positive controls can be made with a known sequence, which, again, can be compared to PCR amplicons of the correct size.

Negative extraction control. This control is used to monitor contamination during nucleic acid extraction and cross-reaction with the host tissue. The control comprises of nucleic acid that is extracted from uninfected host tissue and subsequently amplified. Multiple controls are recommended when large numbers of positive samples are tested.

3.4.2 DNA extraction

DNA can been successfully extracted from lesions or plant material using GeneAll Exgene Plant SV kit according to the manufacturer's protocol (GeneAll) or Cetyl trimethylammonium bromide (Refer to Appendix 2). Prior to DNA extraction, pycnidial stromata of *A.* ulei or ascostromata of *M. ulei* are excised from a single lesion of an infected leaf with a sterilized razor blade. Each lesion is examined under the microscope to check for possible contamination by mycoparasites and selected stromata (approximately 10 structures) are transferred to a microtube (1.5mL). The procedure is repeated from another lesion on the same leaf. To break up the

melanised cell walls, the microtubes containing fungal material (mycelium, pycnidia or ascostromata) are placed in liquid nitrogen and macerated using a micropestle.

3.4.3 Conventional PCR

Several primer pairs are available for diagnosis of *M. ulei*. Hora Junior *et al.* (2014) primers LROR and LR5 target 28s rRNA gene (LSU), ITS1 and ITS4 target the first and second internal transcribed spacer (ITS), NMS1 and NMS2 target the mitochrondrial region of the mtSSU-rDNA, Mcm7-709for and Mcm7-1384rev target the partial sequences of nuclear genes such as the mini-chromosome maintenance protein (MCM7), EF1-728F and EF1-986R target the translation elongation factor 1-alpha (EF-1α), and ACT-512F and ACT-783R target the actin gene respectively. These primers identify strains including *Microcyclus ulei*, *Flusicladium heveae* and *Aposphaeria ulei*.

PCR protocol of Hora Junior *et al.* (2014)

The primers are:

ITS1 (Forward): 5'- TCCGTAGGTGAACCTGCGG- 3'
ITS4 (Reverse): 5' – TCCTCCGCTTATTGATATGC- 3'

LROR (Forward): 5' – ACCCGCTGAACTTAAGC- 3'
LR5 (Reverse): 5'- ACCCGCTGAACTTAAGC- 3'

NMS1 (Forward): 5'- CAGCAGTGAGGAATATTGGTCAATG-3'
NMS2 (Reverse): 5'- GCGGATCATCGAATTAAATAACAT-3'

Mcm7-709 (Forward): 5'- ACIMGIGTITCVGAYGTHAARCC-3'
Mcm7-1384 (Reverse): 5'- GAYTTDGCIACICCIGGRTCWCCCAT-3'

EF1-728 (Forward): 5'- CATCGAGAAGTTCGAGAAGG-3'
EF1-986 (Reverse): 5'- TACTTGAAGGAACCCTTACC-3'

ACT-512F (Forward): 5'-ATGTGCAAGGCCGGTTTCGC-3'
ACT- 783R (Reverse): 5'- TACGAGTCCTTCTGGCCCAT-3'

The Polymerase Chain Reaction (PCR) mixture is prepared in a sterile tube and consists of 5X MyTaq Buffer, 10pmol/µL of each primer and 5u/µL of Taq DNA polymerase as described by the manufacturer (Bioline). Extracted DNA sample volume of 3 µL is added to 47 µL of the PCR mixture to give a total of 50 µL per reaction. The reaction conditions are an initial denaturation step of 94˚C for 5 min, followed by 35 cycles of 30s at 94 ˚C, 30s at 60 ˚C and 1 min at 72 ˚C, with a final extension of 10 min at 72 ˚C.

3.4.4 Interpretation of results from conventional PCR

The pathogen-specific PCR will be considered valid only if the below criteria are met:
- The positive control produces the correct size amplicon for the fungi i.e 600bp for ITS & 900bp for LSU.
- No amplicons of the correct size for the fungi are produced in the negative extraction control and the negative amplification control

If 18S rDNA internal control primers are also used, then the negative (healthy plant tissue) control (if used), positive control, and each of the test samples will produce an approximately 1.6 kilobase (kb) band (amplicon size will depend on which 16s rDNA primers are used. Note that synthetic and plasmid positive controls will not produce a 1.6 kb band. Failure of the samples to amplify with the internal control primers suggests, for example, that the DNA extraction has failed, the nucleic acid has not been included in the reaction mixture, compounds inhibitory to PCR are present in the DNA extract, or the DNA has degraded.

- A sample will be considered positive if it produces an amplicon of the correct size of 600bp for ITS & 900bp for LSU.

3.5 Identification

Identification of presumptive *Microcyclus ulei* should be verified by two or more techniques. In addition to observing morphological characteristics on media, molecular testing should be performed for confirmation.

3.5.1 PCR methods

It is recommended that in addition to the PCR protocol described in section 3.4.3, the identification of pure cultures of suspect strains is confirmed by using two different sets of primers. This is because of the findings that currently, most published primer pairs lack specificity. The primer pairs of Hora Junior *et al.* (2014) have been found to be specific to extracted DNA from pure cultures but not so for extracted DNAs from suspected infested leaf tissues.

Identification can be further confirmed by sequencing the resulting PCR amplicons and comparing their sequences with those of *Microcyclus ulei* deposited in the European Bioinformatics Institute (EMBL-EBI) GenBank database.

3.5.2 Sequence analysis

PCR products should be sequenced either directly or by first cloning them into a PCR cloning vector. Sequence data can be analysed using the Basic Local Alignment Search Tool, BLAST, available at the European Bioinformatics Institute (EMBL-EBI) GenBank (http://www.ebi.ac.uk/Tools/sss/fasta/nucleotide.html). If the sequence shares less than 97.5%

identity with its closest relative, the *Microcyclus* is considered to be a new '*Microcyclus ulei'* species. Sequencing a separate region of the genome such as the mitochondrial region of the mtSSU-rDNA, mini-chromosome maintenance protein (MCM7), translation elongation factor 1-alpha (EF-1α) and actin gene is also desirable.

3.6 Records

Records and evidence should be retained as described in ISPM 27 (*Diagnostic protocols for regulated pests*).

In cases where other contracting parties may be affected by the results of the diagnosis, in particular in cases of non-compliance and where the *Microcyclus* is found in an area for the first time, the following records and evidence and additional material should be kept for at least one year in a manner that ensures traceability:

- The original sample, kept frozen at -80˚C, or freeze-dried or dried over calcium chloride and kept at 4˚C.
- If relevant, DNA extractions should be kept at -20˚C or at -80˚C. Plant extracts spotted on membranes should be kept at room temperature.
- If relevant, PCR amplification products should be kept at -20˚C or at -80˚C.

4 References

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Appendix

Appendix 1. Operative Method for preparation of the culture medium for *M.ulei*

Include the following components to prepare 1 litre of culture medium for the isolation and growth of mycelium for *M.ulei*

- Boil 250grams of peeled potato in 700ml of distilled water
- Prepare 200ml of distilled water of the following product
  - 10grams of Sucrose
  - 2grams of Monobasic Potassium Phosphate
  - 0.15grams of Chloramphenicol
- Pass the potato broth through a fine sieve after cooking
- Cool the potato broth and add the reagents as mentioned in Step 2
- Add the following amino acids in a laminar flow hood
  - 1ml of lysine hydrochloride at a concentration of 10,000ppm
  - 0.1ml of Tryptophan at the concentration of 2,500ppm
  - 0.1ml of Threonine at a concentration of 2,500ppm
- Adjust the solution to pH 5.0± 0.2 with 1N of HCl or NaOH
- Add 20grams of Agar and dissolve in the microwave oven
- Transfer the medium to a 1000ml beaker and make up to volume with distilled water
- Transfer the hot medium to test tubes and autoclave at 121˚C at 15mins
- Tilt the tubes to solidify and store in the refrigerator after autoclaving

Appendix 2. High quality genomic DNA extraction using CTAB

- Prepare the following buffers prior to extraction of DNA
  - Buffer A: 0.35 M Sorbitol, 0.1 M Tris-HCl pH 9 and 5mM EDTA pH 8
  - Buffer B: 0.2 M Tris-HCl pH 9, 50mM EDTA pH 8, 2M NaCl and 2% CTAB
  - Buffer C: 5% Sarkosyl (N-lauroylsarcosine sodium salt Sigma L5125)
  - Lysis buffer for 17.5ml: 2.5 vol of Buffer A, 2.5 vol of Buffer B, 1.0 vol of Buffer C, 0.1% PVP and Proteinase K.
- Pre-warm Buffer B at 65 ˚C
- Prepare the lysis buffer using 17.5ml per 500mg of starting material
- Grind tissue using liquid nitrogen in a mortar, and transfer 500mg of finely ground powder in a 50ml Falcon tube

- Add 17.5ml of lysis buffer to the tube and mix by vortexing
- Incubate for 30min at 65 ˚C and mix frequently by inverting the tube
- Add 5.75ml (0.33vol) of KAc (5M), mix by inverting the tube and incubate on ice for 30mins
- Centrifuge for 20min at 4,000g at 4 ˚C
- Transfer the supernatant in a new 50ml Falcon tube and add 1 vol of Chloroform: Isoamyl alcohol (24:1)
- Centrifuge for 10min at 4,000g at 4 ˚C
- Transfer the aqueous phase in a 50ml centrifuge tube
- Add 100µl of RNAse A (10mg/ml) and incubate for 90- 120min at 37 ˚C
- Add 1/10 vol of NaAc and 1 vol of isopropanol (room temperature) and incubate for 5min at room temperature
- Centrifuge for 30min at 10,000g at 4 ˚C
- Discard the supernatant
- Wash the pellet with 2ml of ethanol (70%) and centrifuge for 10 min at 10,000g at 4 ˚C
- Discard the supernatant and dry the pellet for 5min at room temperature
- Resuspend the pellet in 500µl of TE at 65 ˚C and store at -80 ˚C

Appendix 3: Synthesized SALB gene (ITS and 28s rRNA)

GTAAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACCGAGCGAGGGCCGCCGCCC
GACCTCCAACCCCTCATGTGAACCCCACCCGTTGCTTCGGGGGCGACCCCGCCGGCACGCGTGCCGGCGCC
GGGCGCCCCCGGAGGTCTCCTCAACACGGCATCTTCTGCGTCGGAGCGTCACAGCAAATGACACAAAACTTT
CAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG
AATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCGAAGGGCATGCCTGTTCGAG
CGTCATTACACCCCTCAAGCCCCGGCTTGGTGTTGGGCGTCGCGGCCCCGCGCGCCTCAAAGTCTCCAGCCA
GGCCGTCCGTCTCCTAGCGTCGTGCAGTCACATCATCCGCTTTGGAGCGCGGGCGGCCGCGGCCGTCAAAC
CGTCCACTCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA
AAGAAACCAACAGGGGATTGCCCTAGTAACGGCGAGTGAAGCGGCAACAGCTCAAATTTGAAATCTGGCGT
CAGCCCGAGTTGTAATTTGTAGAGGATGCTTCCGGGCAGCGGCCGGTCTAAGTTCCTTGGAACAGGACGTC
ACAGAGGGTGAGAATCCCGTACGTGACCGGCTTGCACCCTCCGCGTAGCTCCTTCGACGAGTCGAGTTGTTT
GGGAATGCAGCTCTAAATGGGAGGTAAATTTCTTCTAAAGCTAAATACCGGCCAGAGACCGATAGCGCACA
AGTAGAGTGATCGAAAGATGAAAAGCACTTTGGAAAGAGAGTTAAACAGCACGTGAAATTGTTGAAAGGG
AAGCGCCCGCAACCAGACTTTGCGGCGGCGTTCGCCCGGGGTTCTCCCCGGGTCACTCGCCGCCGCGAGGC
CATCATCGTCCGGGACCGCTGGATAAGACCCGAGGAATGTGGCTTCCCTCGGGAAGTGTTATAGCCTCGGG
TGATGCAGCGCGTCTCGGGCGAGGTCCGCGCCCCGGCAAGGATGATGGCGTAATGGTTGTCGGCGGCCCG
TCTTGAAACACGGACCAAGGAGTCTAACATCTATGCGAGTGTTCGGGTGTCAAACCCCTACGCGTAATGAAA
GTGAACGGAGGTGGGAGCTTCTCGCGCACCATCGACCGATCCTGATGTCCTCGGATGGATTTGAGTAAGAG
CATAGCTGTTGGGACCCGAAAGATGGTGAACTATGCCTGAATAGGGTGAAGCCAGAGGAAACTCTGGTGG
AGGCTCGCAGCGGTTCTGACGTGCAAATCGATCGTCAAATTTGGGTATAGGGGCGAAAGACTAATCGAACC
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ACTTTATTGAACGTGGACATTTGAATGCACCGTTACTAGTGGGCCATTTTTGGTAAGCAGAACTGGCGATGC
GGGATGAACCGAACGCGAGGTTAAGGTGCCGGAATGTACGCTCATCAGACACCACAAAAGGTGTTAGTTCA

TCTAGACAGCAGGACGGTGGCCATGGAAGTCGGAATCCGCTAAGGAGTGTGTAACAACTCACCTGCCGAAT
GAACTAGCCCTGAAAATGGATGGCGCTCAAGCGTACTACCCATACCTCGCCGCCAGGGTAGAAACGATGCC
CTGGCGAGTAGGCAGGCGTGGGGGCTCGTGACGAAGCCTTCGGAGTGATCCGGGGTCGAACAGCCTCTAG
TGAGAC