National Diagnostic Protocol

Phymatotrichopsis omnivora

The cause of Texas root rot

NDP 50 V1

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Purpose

National Diagnostic Protocols (NDPs) are diagnostic protocols for the unambiguous taxonomic identification of plant pests. NDPs:

- are a verified information resource for plant health diagnosticians
- are consistent with ISPM No. 27 – Diagnostic Protocols for Regulated Pests
- provide a nationally consistent approach to the identification of plant pests enabling transparency when comparing diagnostic results between laboratories; and,
- are endorsed by regulatory jurisdictions for use (either within their own facilities or when commissioning from others) in a pest incursion.

Where an International Plant Protection Convention (IPPC) diagnostic protocol exists, it should be used in preference to NDPs although NDPs may contain additional information to aid diagnosis. IPPC protocols are available on the IPPC website:

https://www.ippc.int/core-activities/standards-setting/ispms

Process

NDPs are facilitated and endorsed by the Subcommittee on Plant Health Diagnostics (SPHD). SPHD reports to Plant Health Committee and is Australia's peak technical and policy forum for plant health diagnostics.

NDPs are developed and endorsed according to Reference Standards developed and maintained by SPHD. Current Reference Standards are available at https://www.plantbiosecuritydiagnostics.net.au/work/subcommittee-on-plant-health-diagnostics/

NDPs are living documents. They are updated every 5 years or before this time if required (i.e. when new techniques become available).

Document status

This version of the National Diagnostic Protocol (NDP) for *Phymatotrichopsis omnivora* is current as at the date contained in the version control box below.

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Further information

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Contents

1 INTRODUCTION 2

2 TAXONOMIC INFORMATION 3

3 DETECTION 4

3.1 Symptoms 4

3.1.1 Cotton and lucerne 5

3.1.2 Woody trees and vines 8

3.2 Sampling 12

4 IDENTIFICATION 13

4.1 Morphological identification 13

4.2 Molecular identification 16

5 CONTACTS FOR FURTHER INFORMATION 19

6 ACKNOWLEDGEMENTS 20

7 REFERENCES 21

8 APPENDICES 23

9 DIAGNOSTICS PROCEDURES TO SUPPORT SURVEILLANCE 24

9.1 Introduction 24

9.2 Sampling 24

9.3 Sample storage and transport 24

9.4 In Field Tests 25

9.5 Laboratory Tests 25

9.6 Acknowledgements 25

9.7 References 25

1 INTRODUCTION

Texas Root Rot (TRR) also known as Cotton Root Rot (CRR) is caused by the soil-borne ascomycete *Phymatotrichopsis omnivora* (Duggar) Hennebert (Marek *et al*. 2009). This fungus does not cause disease in monocotyledonous plants; however, it has potential to impact a huge range of dicotyledonous crops in Australia.

P. omnivora has a broad host range of over 2000 species of dicotyledonous plants including 107 economically important agricultural and horticultural crops, several tree species and ornamental plants (Blank 1953, CAB International 2007, Olsen 2015). The major hosts are cotton (including *Gossypium herbaceum, G. hirsutum*, and *G. barbadense),* lucerne (*Medicago sativa*), woody fruit and nut trees (e.g. almond (*Prunus dulcis*), pecan (*Carya illinoinensis*), walnut (*Juglans regia*), apple (*Malus domestica*), European pear (*Pyrus communis*), peach (*Prunus persica*), fig (*Ficus carica*) and grapevine (*Vitis vinifera*), which are included in this protocol.

TRR also affects other crops such as legumes including peanut (*Arachis hypogaea*), soyabean (*Glycine max*) and various beans (*Phaseolus* spp.); okra (*Abelmoschus esculentus*) and other Malvaceae including kenaf (*Hibiscus cannabinus*); parsley (*Petroselinum crispum*) and other (*Apiaceae*) and sugarbeet (*Beta vulgaris var. saccharifera*). While symptoms seen on these plants are not included in this protocol, the identification techniques will be the same.

The fungus colonises the tap root, causing rapid decay and girdling of the outer periderm and cortical tissues and blocking the flow of water through the vascular cambium. Plants consequently wilt, become chlorotic, and then die. The rate of disease onset differs between plant species. Disease onset is sudden in vegetables and field crops, but the development of extensive root decay in tree and fruit crops can often take up to two seasons (CAB International 2007).

The diagnosis of TRR is relatively straight forward due to the distinctive morphological characteristics of the pathogen. Diagnosis may be based on indicators *in planta* along with pathogen morphology of isolated cultures. However molecular techniques should also be utilised, as the success rate of isolating the fungus into culture can be relatively low and therefore early infections can go undetected.

2 TAXONOMIC INFORMATION

Phymatotrichopsis omnivora (Duggar) Hennebert was previously assumed to be a member of Basidiomycota with possible teleomorphs *Sistotrema brinkmanii* and *Phanerochaete omnivora*.

However, phylogenetic analyses of the nuclear small- and large-subunit ribosomal DNA and subunit 2 of RNA polymerase II from multiple isolates have shown this to be incorrect. *P. omnivora* is an anamorphic member of Ascomycota for which no teleomorph has been described (Marek *et al*. 2009). Synonyms for *P. omnivora* include *Phymatotrichum omnivorum* Duggar, *Ozonium omnivorum* Shear, and *Ozonium auricomum* Link.

Kingdom	Fungi
Phylum	Ascomycota
Class	Pezizomycetes
Order	Pezizales
Family	Rhizinaceae
Genus	*Phymatotrichopsis*
Species	*omnivora*

3 DETECTION

The pathogen can be detected in roots of woody trees and taproots and stems of tap-rooted plants.

3.1 Symptoms

Characteristic symptoms used for detection include wilting or dead plants. Infected plants often wilt suddenly in Summer when temperatures are high and when soil temperatures reach 28°C. The upper most leaves wilt within 24 hours followed by wilting of the lower leaves within 72 hours. Permanent wilt occurs on the 3rd day. In vegetables and field crops the entire root system decays within a few days of wilting. In fruit trees, the rot is slower, and trees may die or show signs of wilting over two growing seasons.

Occasionally, the conidial stage develops as a creamy yellow spore mat on the ground near dying plants (Figures 1, 2). Spore mats are not common and may be confused with other fungi that grow over the soil surface or in decaying litter so they should be positively identified before using them in a diagnosis.

Figure 1. *Phymatotrichopsis omnivora* spore mats in USA (Photos: A - S. McBride, Texas A&M; B - N. Goldberg, New Mexico State University Cooperative Extension Service)

Figure 2. *Phymatotrichopsis omnivora* spore mat on lucerne (*Medicago sativa*). © S.D. Lyda, Bugwood.org.

The fungus produces root-like mycelial strands (rhizomorph) that grow through the soil from plant to plant. Strands then grow on roots towards the soil surface. Infected plants die suddenly. Following plant death, small sclerotia (compact masses of thick-walled cells), irregular in shape form in the strands where cells divide and enlarge. Sclerotia are white then change to brown/black colour with age. Sclerotia enable the fungus to survive for several years in the soil. During warm, wet weather, the fungus can form white to tan coloured spore mats on the soil surface, that vary in size. The spore mats are composed of mycelial strands that produce conidia. The conidia are apparently sterile.

3.1.1 Cotton and lucerne

The disease appears in patches in the field (Figure 3), with lucerne often as “fairy rings” (Figure 4). The first symptoms of the disease are sudden wilting of plants with or without chlorosis of the leaves during the summer. The foliage droops, turns brown, and may remain hanging on the branches for a few days before dropping off (Figure 5). At this stage the roots and lower section of the stem are covered with a network of yellow mycelial strands visible to the naked eye (Figure 6 and 15). Discolouration can be seen inside the stem when cut in cross sections (Figure 7).

Figure 3. Patches of cotton plants infected with *Phymatotrichopsis omnivora* in Texas, USA (Photo: K. Kirkby).

Figure 4. "Fairy ring" patches of lucerne infected with *Phymatotrichopsis omnivora* (Uppalapati *et al*. 2009)

Figure 5. Cotton plants infected with *Phymatotrichopsis omnivora,* with brown foliage remaining on the branches, in Texas USA (Photo: K. Kirkby)

Figure 6. Yellow fungal strands of *Phymatotrichopsis omnivora* on a cotton root, in Texas USA (Photo: K. Kirkby)

Figure 7. Discolouration inside a cotton stem infected with *Phymatotrichopsis omnivora* (Photo: K. Kirkby)

3.1.2 Woody trees and vines

Symptoms of various trees are illustrated in Figures 8-14. Because plants may die during the summer for reasons other than infection with *P. omnivora*, it is necessary to examine root tissue for the presence of the fungus.

Disease symptoms usually occur late spring to early autumn. Plant death can be variable and can occur very rapidly in warmer temperatures (Figure 8). Leaves yellow and wilt, sometimes only on one side of the tree. The dead leaves remain firmly attached to the plant – "cling leaf" (Figure 10).

Affected roots are soft and decayed, and the surface of the roots will be covered with the tan to golden-brown mycelium of the fungus (Figure 12). With careful examination under at least 10X magnification, light to dark brown strands or hyphal webs of the fungus can be observed on the root surface. Strands are most easily identified on fresh material; however, strands and cruciform hyphae can still be observed on dried roots.

Figure 8. Bradford pear tree infected with *Phymatotrichopsis omnivora* (left) and same tree nine days later (right) in USA. (Photo: N. Goldberg, New Mexico State University Cooperative Extension Service).

Figure 9. Grapevine infected with *Phymatotrichopsis omnivora* (Photo: S. McBride, Texas A&M).

Figure 10. Pistachio tree infected with *Phymatotrichopsis omnivora* showing characteristic cling leaf symptom (Photo: N. Goldberg, New Mexico State University Cooperative Extension Service).

Figure 11. Pecan tree infected with *Phymatotrichopsis omnivora* (Photo: N. Goldberg, New Mexico State University Cooperative Extension Service).

Figure 12. Pecan tree roots with *Phymatotrichopsis omnivora* hyphal strands (Photo: N. Goldberg, New Mexico State University Cooperative Extension Service).

Figure 13. Olive tree (left) and apple tree (right) infected with *Phymatotrichopsis omnivora*, USA (Photos: S. McBride, Texas A&M).

Figure 14. Lace bark elm tree infected with *Phymatotrichopsis omnivora* (left) (Photo: S. McBride, Texas A&M).

3.2 Sampling

3.2.1 Cotton and Lucerne

For tap rooted plants (e.g. cotton and lucerne), look for plants that are just starting to wilt. The leaves appear wilted, but they are not yet dried up. It is preferable to inspect fields in the morning before the plants wilt from lack of water. Look in areas of the field where plants have been dead for some time.

Dig up the wilted plants. Check to see if some or the entire top of the root is rotted by scraping the root with fingernail or knife. Roots of infected plants will be discoloured (brown, but not white). Check the lateral roots and cut lower root in half and check for any discoloration (not white). Sample plants with discoloured roots.

Using clippers, cut a sample that is 7-10 cm above the root. Cut off root 7 cm below soil line. Keep just the upper root-lower stem portion (about 14 cm).

3.2.2 Woody trees

For woody trees, the roots need to be excavated, which may need equipment such as a back hoe. Take several samples of rotted and discoloured roots on which the outer or cortical tissue still remains attached. The samples should be at least 1cm thick and 10-20 cm long.

3.2.3 Sample storage and transport

Leave soil attached, and store or transport the roots in a plastic bag under refrigeration. Do not add water or wet paper towels. Root samples are best tested within three days of collection. The hyphae may still be able to be examined morphologically on older samples, and used for molecular testing, however the ability to obtain viable isolates through culturing is significantly reduced.

4 IDENTIFICATION

P. omnivora can be easily identified by observation of its morphology.

4.1 Morphological identification

4.1.1 Microscopic examination from infected material

The species is characterised by distinctive branching acicular cruciform hyphae (10-20 µm in diameter) which are not known in any other species of fungi. The hyphae are septate and often form mycelial strands (rhizomorph). Hyphal strand cells divide, grow and enlarge to form irregularly shaped sclerotia (1-2 mm in diameter). The sclerotia are firstly white changing to brown to black colour with age. The fungal strands also produce conidia, which are found in spore mats associated with symptomatic plants. Conidia are unicellular, hyaline, globose or ovate (4.8 to 5.5 µm in diameter).

- Once at the lab place a drop of deionized water onto a microscope slide
- Using tweezers remove some of the brown/yellow mycelium strand from the root sample and place on the drop of water on the microscope slide (Figure 15)
- Cover with a cover slip
- Examine under a compound microscope, looking for the characteristic hyphal formation of cruciform hyphae (Figure 16) which are easy to find

Figure 15. Removing mycelial strand from infected cotton root (Photo: K. Kirkby)

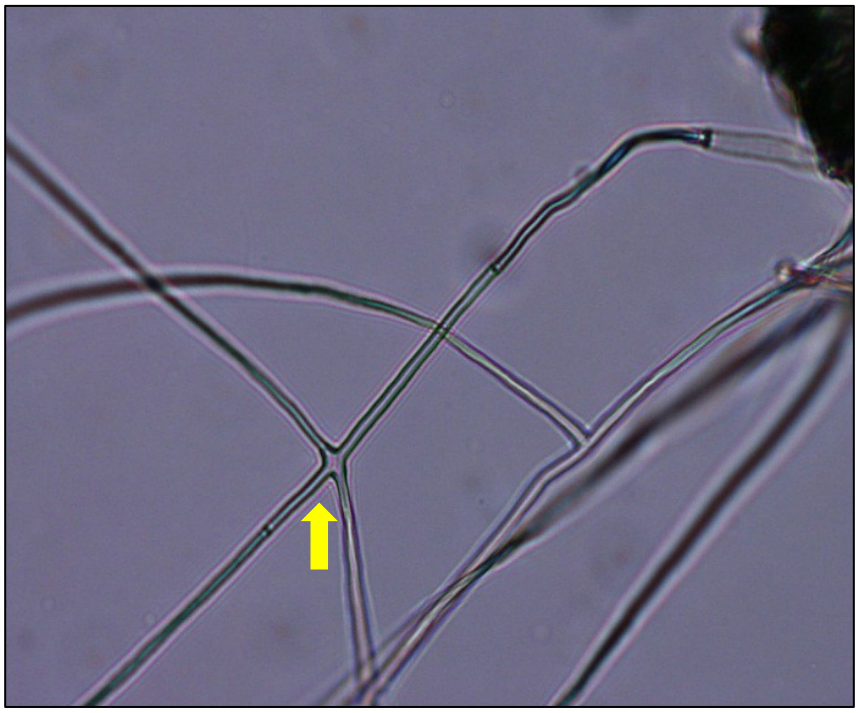

Figure 16. Cruciform hyphae of *Phymatotrichopsis omnivora* (mag x 40) marked by arrow (Photo K. Kirkby)

4.1.2 Isolation of P. omnivora from infected material

This organism can be somewhat difficult to isolate. Reported viability of mycelial strands (i.e., success rate of isolation from mycelial strand segments) ranges from 10-61%, with decreasing success from older strands (Wheeler & Hine 1969). It is easier to isolate the pathogen from infected plant tissue rather than plating individual strands. The pathogen may be isolated by washing segments of strands in water and plating on antibiotic-amended agar. The following media have been used to successfully isolate *P. omnivora*:

1. Water agar with 50 ppm streptomycin sulfate (Cook & Riggs, 1993)
2. Water agar or PDA with 100 ppm each of chloramphenicol, novobiocin bacitracin, and penicillin G (Wheeler & Hine, 1969)
3. 1/5 strength Difco potato dextrose agar with 100 µg/ml streptomycin

Method:

- Remove lateral roots from the samples with clippers and discard
- Wash sample under running water. Remove soil with fingers or brush
- Copiously spray middle 10 cm of root/stem section with 70% ethanol until it runs off
- Blot dry with clean paper towel and place the sample on clean paper towel
- Hold stem at top end and use a sterile scalpel to peel outer part of stem, peeling away from you
- Peel close to root/stem interface OR look for where the root is browner than the rest
- Peel and cut out a piece (3 x 3 mm) at the brown area that is about 1 mm thick
- Place a piece on Petri plate of agar, about 6 mm from edge of the plate. Push the piece firmly onto the agar.
- Repeat by taking three additional pieces from the same area of root or from nearby.
- Cover the Petri plate with lid and place in a sealed plastic bag. This will keep the agar from

drying out during the incubation at high temperature.
- Incubate at 30˚C ±1˚C for 4 days

Results:

Colony growth: floccose strands (consisting of or covered with woolly tufts) growing flat on agar after 48 hours, hyphae often matted or compacted, yellow to deep orange to brown, forming aerial cruciate hyphae (Figure 17).

Figure 17. Four-day old colony growth of *Phymatotrichopsis omnivora* on 1/5 strength potato dextrose agar (Photo K. Kirkby)

4.2 Molecular identification

The PCR and qPCR protocols for the identification of *P. omnivora* have been reproduced with permission from published methods (Arif *et al.* 2013 & 2014). Cycling conditions specified in these papers have been modified to improve performance.

4.2.1 DNA extraction

Any standard fungal/plant DNA extraction protocol can be used. A recommended protocol for DNA extraction from fungi is the DNAeasy Plant Mini Kit (Qiagen, Valencia, CA). Extract DNA from lyophilized root samples using the MagAttrcat 96 DNA plant kit (Qiagen). Determine DNA concentrations using a spectrophotometer such as a NanoDrop v.2000 spectrophotometer (Thermo Fisher Scientific Inc., Worcester, MA).

To permit DNA isolation in the field, a Dynabeads DNA Direct Universal kit (Invitrogen, Carlsbad, CA) can be used with modifications to the manufacturer's instructions. Prepare target *P. omnivora* templates using 10 to 30 mg infected plant root tissues and 100 to 150 µL Tris-EDTA (TE) buffer (Promega, Madison, WI). Macerate samples using an Eppendorf tube and micropestle. Mix 40 µL of macerated supernatant and 200 µL of Dynabeads and incubate for 5 mins. Place tubes containing the macerated supernatant and Dynabead mix in a magnetic rack until the beads form a tight pellet, then carefully remove the supernatant and discard and rinse twice with wash buffer. Finally, add 40 µL of kit suspension buffer to the washed beads to release and suspend isolated DNA.

4.2.2 Controls

For conventional and real-time PCR, a plant housekeeping gene such as COX1 (Weller et al., 2000), 16S ribosomal (r)DNA (Weisberg et al., 1991) or GADPH (Mafra et al., 2012) should be incorporated into the protocol or run as a separate test as internal control. This is to eliminate the possibility of PCR false negatives due to nucleic acid extraction failure, nucleic acid degradation or presence of PCR inhibitors.

For the test result to be considered reliable, appropriate controls should be considered and used for each stage of testing. For PCR, a positive control (plasmid DNA carrying the target gene, genomic DNA, or synthetic oligonucleotides), an internal control, a negative amplification control (no template control) and a negative extraction control are the minimum controls that should be used.

4.2.3 End point PCR (Arif et al. 2014)

Amplification

Carry out amplifications for the primer set (Table 1) in 20 µL reaction mixtures containing:

- 10 µL of GoTaq Green Master Mix (Promega) or any standard PCR Master Mix
- 1 µL (5 µM) of each forward and reverse primer
- 1 µL bovine serum albumin (BSA) (10 mg/mL)
- 2 µL of template genomic DNA
- 6 µL of nuclease-free water

Parameters for thermocycler are as follows:

- Initial denaturation 3 min at 94°C
- 35 cycles of denaturation at 94°C for 30s
- Annealing at 60°C for 30s
- Extension at 72°C for 30s
- Final extension at 72°C for 3 min

Table 1. End point PCR primers (Arif *et al*. 2014)

Primer name	Primer sequence (5' – 3')	Target gene region	Amplicon size (bp)
PO2F	AAACCCCCAAATGGATGC	ITS	564
PO2R	CACCACCATACTACGTCAAAGG	ITS	

Use 5 µL of amplified PCR product to electrophorese in a 1.5% agarose gel in 1xTris-acetate –EDTA (TAE) buffer and estimate amplicon sizes using a 100 bp ladder (e.g. Invitrogen).

4.2.4 Real time PCR (Arif et al. 2013)

Amplification

Carry out amplifications for each primer set (Table 2) in 20 µL reaction mixtures containing:

- 10 µL of Platinum Quantitative PCR SuperMix-UDG (Invitrogen)
- 1.2 µL (5 µM) of each forward and reverse primer
- 0.4 µL (5µM) probe
- 0.6 µL bovine serum albumin (BSA) (10 mg/mL)
- 2 µL of template genomic DNA
- 4.6 µL of nuclease-free water (Ambion).

Parameters for thermocycler are as follows:

- Initial hold for 2 min at 50°C
- Initial denaturation for 2 min at 95°C
- 40 cycles of 95°C for 15 s and 60°C for 60 s.

Table 2. Real time PCR primers and double-quencher probes contain a 5′ FAM fluorophore, a 3′ IBFQ quencher, and an internal ZEN quencher to maximize signal and minimize background. (Arif et al. 2013).

Primer name	Primer sequence (5' – 3')	Target gene region	Amplicon size (bp)
PO4F	GTTCGAGCGTCAGCATAACA	ITS2-5.8S rDNA	116
PO4 R	AAGACACCACCCATACATTTCAG	ITS2	
PO4 probe	TGGCTTGGTCATTGGCGGTG	ITS2	

PObt1 F	GGTCTTGATGGTTCTGGTGTGT	Beta-tubulin	126
PObt1 R	TCCAACTGGAGGTCAGAGGTA	Beta-tubulin	
PObt1 probe	TCCCCATAGAGCCGATAGAGTGCTG	Beta-tubulin	
PORPB2-2F	GTGTCTGCCGTCCACTTTTC	RPB2	135
PORPB2-2R	CCATCCGAACCTCTCCTCTT	RPB2	
PORPB2 probe 2	ATAACGACCCGACGAGCGAGAGG	RPB2	

4.2.5 Interpretation

End point PCR

Sequencing of the amplicon is necessary. The PCR test is **negative** if the amplicons are not of expected size or no bands appear for the sample in question, while an amplicon is detected for all positive control samples.

The PCR test is **positive** if the specific amplicon of expected size is detected, and if it is not amplified from any of the negative amplification or extraction control samples.

Inhibition of the PCR may be suspected if no amplification is obtained for the positive control or internal control samples.

When the results are inconclusive:

a) repeat the test to rule out contamination

b) sequence the PCR product.

It is necessary to use the multigene approach to maximise reliability, specificity and broad range detection within the P. omnivora variants by minimising the risk of false positives and false negatives because each gene acts as an internal control for the two other genes (Arif et al. 2013).

Real-time PCR

The real-time PCR test results are considered valid only if:

- the positive control produces an amplification curve with the pathogen-specific primers

- no amplification curve is produced with the pathogen-specific primers for the negative extraction and amplification controls.

A sample will be considered positive if it produces a typical amplification curve. The acceptable Ct value needs to be verified in each laboratory when implementing the test for the first time.

If the COX 1 internal control primers are used, then each of the test samples must produce an amplification curve. Failure of the samples to produce an amplification curve with the internal control primers suggests, for example, that the DNA extraction failed, the DNA was not included in the reaction mixture, PCR inhibitory compounds were present in the DNA extract, or the nucleic acid was degraded.

The real time PCR assay targets three fungal genes at a time. The Cq values are expected to be different for these genes as ITS is a multi-copy gene and the beta-tubulin and RPB2 genes are single copy.

In case of low fungal DNA concentration, it is possible that only one probe (targeting ITS) can detect *P. omnivore* as the ITS is a multi-copy gene.

5 CONTACTS FOR FURTHER INFORMATION

The Cotton Pathology Group of NSW Primary Industries located at the Australian Cotton Research Institute (ACRI), Narrabri is the preferred diagnostic laboratory to process plants suspected of Texas or Cotton Root Rot. This laboratory has developed expertise in the detection and diagnosis of this disease through visiting Texas, USA where the disease is prevalent. Photos should be taken of suspected plants and sent as soon as possible to the laboratory, along with the location (GPS) of suspected infection and contact information.

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The protocol was reviewed by Merje Toome and verified by Ruth Griffin, Plant Health & Environment Laboratory, Diagnostic and Surveillance Services, Ministry for Primary Industries, Auckland New Zealand.

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8 APPENDICES

Not applicable

9 DIAGNOSTICS PROCEDURES TO SUPPORT SURVEILLANCE

9.1 Introduction

Texas Root Rot (TRR) caused by the soil-borne ascomycete *Phymatotrichopsis omnivora* infects dicotyledonous crops. TRR has a broad host range of over 2000 species including 107 economically important agricultural and horticultural crops, several tree species and ornamental plants (Blank 1953, CAB International 2007, Olsen 2015). The major hosts are cotton, lucerne, woody fruit and nut trees, pecan, walnut, apple, European pear, peach, fig, grapevine, peanut, soyabean and various beans. Symptoms include root decay, wilting, chlorosis and plant death.

Testing for TRR include common laboratory methods such as isolation and morphological identification and molecular testing using PCR (Table 1). Molecular techniques are the preferred method as the success rate of isolating the fungus into culture can be relatively low and therefore early infections can go undetected. One method which aids in targeting surveillance/sampling in the field is looking for the wilted plants with the characteristic hyphal mat at the base of infected plants.

Table 1. Methodology required for the identification of TRR

Method	Identification Level	Identification Confidence	Deployment (Field/Lab)	Required Time	Throughput
Morphology	To Genus	Medium	Laboratory	<1 Day	Low (<100)
Molecular	To Species	Medium	Laboratory	< 1 Week	High (>100)

9.2 Sampling

Sampling for TRR should be undertaken in the morning before plants wilt from the lack of water and concentrate in areas of the field where plants have died. Sample plants that show signs of wilting and discoloured (brown, not white) roots. For tap root plants such as cotton, cut stem above the soil line and about 7-10cm below the soil line. Collect several root samples per field. For woody trees, take several samples at least 1cm thick and 10-20cm long of rotted and discoloured roots on which the outer or cortical tissue remains attached.

9.3 Sample storage and transport

Leave soil attached, and store or transport the roots in a labelled ziplock plastic bag. Keep samples in a cool dry place for transport; an esky may be required when sampling when temperatures are hot. Do not add water or wet paper towels into the sample bags. Isolations from root samples are best when done within 3 days of collection. Hyphae may still be able to be examined morphologically on older samples and used for molecular testing.

9.4 In Field Tests

No in-field testing available.

9.5 Laboratory Tests

Laboratory methods for the detection of TRR include morphological and molecular techniques. Both methods including the instructions to complete can be located in the NDP for TRR in sections 4.1 and 4.2 respectively.

9.6 Acknowledgements

This surveillance section was written by Dr Karen Kirkby (NSW DPI)

9.7 References

Marek SM, Hansen K, Romanish M, Thorn RG. 2009. Molecular systematics of the cotton root rot pathogen, *Phymatotrichopsis omnivora*. *Persoonia* 22: 63–74.