National Diagnostic Protocol

Fusarium oxysporum f. sp. *ciceris*

The cause of fusarium wilt of chickpea

NDP36 V1

This publication (and any material sourced from it) should be attributed as: Subcommittee on Plant Health Diagnostics (2016). National Diagnostic Protocol for *Fusarium oxysporum* f. sp. *ciceris* – NDP36 V1. (Eds. Subcommittee on Plant Health Diagnostics) Authors Cunnington, J, Lindbeck, K and Jones, RH; Reviewer Baskarathevan, J. ISBN 978-0-9945113-0-0. CC BY 3.0.

Cataloguing data
Subcommittee on Plant Health Diagnostics (2016). National Diagnostic Protocol for *Fusarium oxysporum* f. sp. *ciceris* – NDP36 V1. (Eds. Subcommittee on Plant Health Diagnostics) Authors Cunnington, J, Lindbeck, K and Jones, RH; Reviewer Baskarathevan, J. ISBN 978-0-9945113-0-0.

ISBN; ISBN 978-0-9945113-0-0

Internet
Report title is available at: http://plantbiosecuritydiagnostics.net.au/resource-hub/priority-pest-diagnostic-resources/

Department of Agriculture and Water Resources

Street Address 1: 18 Marcus Clarke Street, Canberra City ACT 2601
Street Address 2: 7 London Circuit, Canberra City ACT 2601
Postal Address: GPO Box 858, Canberra City ACT 2601
Switchboard Phone: 02 6272 3933
Web: http://www.agriculture.gov.au

Inquiries regarding the licence and any use of this document should be sent to: copyright@agriculture.gov.au.

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 http://plantbiosecuritydiagnostics.net.au/sphd/sphd-reference-standards/

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 *Fusarium oxysporum* f. sp. *ciceris* is current as at the date contained in the version control box below.

PEST STATUS	Not present in Australia
PROTOCOL NUMBER	NDP36
VERSION NUMBER	V1
PROTOCOL STATUS	Endorsed
ISSUE DATE	June 2016
REVIEW DATE	2021
ISSUED BY	SPHD

The most current version of this document is available from the SPHD website: http://plantbiosecuritydiagnostics.net.au/resource-hub/priority-pest-diagnostic-resources/

Further information

Inquiries regarding technical matters relating to this project should be sent to: sphds@agriculture.gov.au

Contents

1 INTRODUCTION

The soil-borne fungus *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo and K. Satô is pathogenically associated primarily with *Cicer* species, of which the high-value pulse crop, chickpea (*Cicer arietinum* L.) is the only cultivated species.

Fusarium oxysporum f. sp. *ciceris* can be spread through infested plant debris (root, leaf and stem), soil and seed as mycelium, microconidia, macroconidia and, most commonly, as chlamydospores.

High pathogenic variability is observed in populations of *F. oxysporum* f. sp. *ciceris.* Symptom type has been used to subdivide *F. oxysporum* f. sp. *ciceris* into two pathotypes (Trapero-Casas and Jiménez-Díaz 1985), designated wilting pathotype and yellowing pathotype. Pathotypes are assigned to pathogenic races according to variation in virulence. The *Fusarium oxysporum* f. sp. *ciceris* races can be defined by differential disease reaction on chickpea genotypes. Eight races of *F. oxysporum* f. sp. *ciceris* with distinct geographic distributions have been identified (Jiménez-Gasco *et al*. 2002).

1.1 Primary host range

Table 1. Host range of *Fusarium oxysporum* f. sp. *ciceris*

Host	**Reference**
Cicer arietinum (chickpea)	Haware *et al*. (1986), Nene *et al*. (1996)
Cajanus cajan (pigeonpea)	Haware and Nene (1982)
Lens culinaris ssp. *culinaris* (lentil)	Haware and Nene (1982)
Pisum sativum (field pea)	Haware and Nene (1982)

Cicer arietinum (chickpea) is the only known seed borne host of *F. oxysporum* f. sp. *ciceris* (Haware *et al*. 1986, Richardson 1990).

2 TAXONOMIC INFORMATION

Kingdom: Fungi
Phylum: Ascomycota
Subphylum: Pezizomycotina
Class: Sordariomycetes
Subclass: Hypocreomycetidae
Order: Hypocreales
Family: Nectriaceae
Genus: *Fusarium*
Species: *oxysporum*

Fusarium oxysporum f. sp. *ciceris* (Padwick) Matuo and K. Satô (as *'ciceri'*), Trans. Mycol. Soc. Japan 3: 125 (1962)

Synonyms

Fusarium lateritium f. sp. *ciceris* (Padwick) Erwin
Fusarium merismoides f. sp. *ciceris* (Padwick) Subram
Fusarium orthoceras var. *ciceris* Padwick

Common names

Fusarium wilt of chickpea
Wilt of chickpea
Wilt of pigeonpea

3 DETECTION

3.1 Stages of development

Following infection of host roots, the fungus crosses the cortex and enters the xylem tissues. It then spreads rapidly upward through the vascular system, becoming systemic in the host tissues, and may directly infect the seed. Seed infestation and infection is common. *Fusarium oxysporum* f. sp. *ciceris* can be internally seed borne and the pathogen is found as chlamydospore-like structures in the hilum region of the seed. Movement of infected seed plays an important role in the long distance dispersal and transmission of fusarium wilt diseases into new areas.

The root tips of healthy plants growing in contaminated soil are penetrated by the germ tube of spores or the mycelium. Entry is either direct, through wounds, or opportunistic at the point of formation of lateral roots. The mycelium takes an intercellular path through the cortex, and enters xylem vessels through the pits. The pathogen is primarily confined to the xylem vessels in which the mycelium branches and produces microconidia. The microconidia detach and are carried upward in the vascular system until movement is stopped, at which point they germinate and the mycelium penetrates the wall of the adjacent vessel. Lateral movement between vessels is through the pits.

The water economy of infected plants is eventually severely compromised by blockage of vessels, resulting in stomatal closure, wilting and death of leaves, often followed by death of the whole plant. The fungus then invades all tissues of the plant to reach the surface where it sporulates profusely. Spores may then be dispersed by wind, water or movement of soil or plant debris. *Fusarium oxysporum* f. sp. *ciceris* can survive as mycelium and chlamydospores in seed and soil, and also on infected crop residues, roots and stem tissue buried in the soil for up to six years (Singh *et al*. 2007). Chlamydospores can survive in soil either in dormant form or saprophytically without a suitable host. The disease is favoured by warm and dry soil conditions with an optimal temperature of 22-25°C.

3.2 Symptom description

***Fusarium oxysporum* f. sp. *ciceris* (races 1A, 2, 3, 4, 5 and 6) - Wilting pathotype.**

Flaccidity of leaves and succulent shoots, followed by discoloration and chlorosis of leaves, desiccation and death; vascular (xylem) and pith tissues show discoloration, usually evident in cross sections of stem near the base.

***Fusarium oxysporum* f. sp. *ciceris* (races 0 and 1A/B) - Yellowing pathotype.**

Progressive foliar yellowing from the base upwards; abscission of necrotic leaves; vascular (xylem) and pith tissues show discoloration.

Figure 1 shows typical distribution of chickpea plants infected with *F. oxysporum* f. sp. *ciceris* under field conditions. Careful examination of internal tissue of infected roots (**Figure 2**) can differentiate fusarium wilt from other root diseases of chickpea seedlings. Wilt can be observed within 25 days of sowing seed into infected soil (Nene *et al*. 1978). Affected seedlings show drooping of the leaves and are a dull green colour. Seedlings may collapse and lie flat on the ground and, when uprooted, may show uneven shrinkage around the collar at the base of the stem. The roots do not show any external

rotting and look apparently healthy. When split vertically from the collar region downward, such roots show a brown discolouration of the internal tissues. This combination of symptoms is unique to fusarium wilt. Infection by virus or phytophthora root rot will produce similar, but not identical symptoms.

Wilting may also occur in adult plants up until the reproductive and podding stage. Drooping of the petioles, rachis and leaflets in the upper part of the plant, together with the pale green colour of the foliage, are the most common symptoms. Often within two to three days the entire plant is affected (Haware *et al*. 1986). Lower leaves also become chlorotic. When uprooted before completely dried, affected plants show no external root discolouration. However, internal discolouration may be seen extending up towards the stem. Internal discolouration is due to infection of the xylem tissues of the root and stem. Transverse sections of the infected root examined under the microscope show the presence of hyphae and spores of the fungus in the xylem. This is a diagnostic feature of fusarium wilt. In certain chickpea cultivars typical symptoms may not develop. Instead, there is yellowing and drying of the lower leaves, and a stunting of the plant. Roots will show internal discolouration. **Figure 3** shows typical yellowing symptoms.

While the affected plant is alive the pathogen is confined to the vascular system and possibly a few surrounding cells. At plant death, the fungus moves to other tissues and sporulates at or near the plant surface. Plants grown from infected seed develop wilt symptoms faster than plants originating from clean seed.

Figure 1. Typical distribution of chickpea plants infected by *Fusarium oxysporum* f. sp. *ciceris* under field conditions in Syria (Photo K. Lindbeck 2002).

Figure 2. Transverse section of a chickpea tap root showing internal discoloration caused by *Fusarium oxysporum* f. sp. *ciceris* infection at ICARDA, Syria. (Photo B. Bayaa ICARDA 2002)

Figure 3. An uprooted chickpea plant affected by fusarium wilt, clearly showing typical yellowing symptoms at ICARDA, Syria. (Photo K. Lindbeck 2002)

3.3 Sites of infection

Predominantly roots and stems, but also leaves, pods and seeds.

3.4 Other root diseases of chickpeas

There are several common root diseases of chickpea in Australia; these include phoma blight (*Didymella pinodella* syn. *Phoma medicaginis* var. *pinodella*), damping off (*Pythium* spp.), rhizoctonia root rot (*Rhizoctonia* spp.) and phytophthora root rot (*Phytophthora medicaginis*). Fusarium wilt rot of chickpea may easily be mistaken for one of these common root diseases, but there are several key symptoms that are unique to this disease (see - section 3.2).

4 IDENTIFICATION

Diagnosis of fusarium wilt of chickpea involves several stages. Firstly, fungal culturing and microscopic examination is undertaken to determine whether *Fusarium oxysporum* can be isolated from the affected plant. If *F. oxysporum* is isolated from the affected plant then a specific PCR (Jiménez-Gasco and Jiménez-Díaz 2003) can be used to determine if the isolate is *Fusarium oxysporum* f. sp. c*iceris.* This PCR has been validated against all 8 races of *Fusarium oxysporum* f. sp. c*iceris* listed in 3.2. Given that this PCR could fail to detect new or previously unrecognised races, host species and seedling pathogenicity tests (see Appendix 8.3) are recommended when an incursion or outbreak is suspected.

4.1 Isolation of organism

Standard fungal isolation methods can be used to isolate *F. oxysporum* from diseased plant material. An example is given below. Equivalent in house methods are acceptable.

4.1.1 Laboratory requirements

- Sterile instruments (scalpel, forceps)
- Tissue
- Marking pen
- 0.5% Sodium hypochlorite (NaOCl)
- Sterile distilled water (SDW)
- 500 mL container (e.g. takeaway food container)
- 100 mL beakers
- Crucible (suitable size to fit in 100 mL beaker)
- Plates of Potato Dextrose Agar + Acromycin (PDAA) and Water Agar (WA)

4.1.2 Isolation procedure

- Thoroughly wash all soil and debris from plant parts
- Place selection of plant parts in container with water
- Select plant parts to be sampled, cut into 3-5 mm segments, place cut segments into crucible (in beaker of water)
- Place crucible with plant segments into beaker containing 0.5% NaOCl for approximately 1 minute, depending on size of segments
- Place crucible with treated plant segments into beaker containing SDW, agitate gently
- Transfer crucible with plant segments into second beaker containing SDW, agitate gently
- Empty contents of crucible onto clean tissue and remove excess water from plant segments
- Evenly space seven separate plant segments onto the agar surface of each plate. Plate representative plant segments onto both types of agar (PDAA and WA).
- Incubate at 20°C until adequate fungal growth is observed

4.2 Morphological identification of *Fusarium oxysporum*

Fusarium oxysporum isolates should be recognized relatively easily in a plant pathology laboratory. On PDA most isolates produce typical fusarium colonies, microconida in false heads on short monophialides, and chlamydospores. Often these features can be quickly observed by placing the plate onto the stage of a compound microscope and examining using a 10x objective. A more detailed morphological description is given in Appendix 8.2.

4.3 Molecular identification method (PCR)

The PCR test needs to be performed using pure cultures of *Fusarium oxysporum* isolated from plant material. This PCR protocol was independently verified in another laboratory (Appendix 8.1). This test was not designed nor validated for directly testing diseased plant material.

4.3.1 Laboratory requirements

- Protective gloves
- Pipettes (2.0, 20 and 300 µL) and sterile plugged tips
- Microcentrifuge and microcentrifuge tubes (1.5 mL)
- PCR tubes (0.2 mL)
- PCR reagents (individual PCR ingredients or a commercial PCR mastermix)
- Thermocycler
- Gel tray with suitable comb/s, electrophoresis tank and powerpack
- Agarose
- Gel running buffer (eg. 0.5× TBE or 1% TAE)
- Suitable size DNA Ladder
- Gel loading dye
- UV transilluminator
- Camera/gel documentation system

4.3.2 Extraction of DNA

Fungal cultures are grown on PDA until there is adequate material for DNA extraction using a standard DNA extraction method such as DNeasy® Plant Mini Kit (Qiagen) as per the manufacturer's instructions. Other equivalent in house methods are acceptable.

4.3.3 DNA quality testing PCR

A PCR using elongation factor primers EF1 and EF2 (**Table 2**) is undertaken to evaluate the quality of DNA extracted from fungal culture. This single copy gene region is harder to amplify than the rDNA ITS region, thus serves as a better test of DNA quality. (See Appendix 8.1.2)

Table 2. Primer sequences used in PCR to evaluate quality of *Fusarium* DNA (O'Donnell *et al*. 1998).

Primer	Sequence
EF1	5'-ATGGGTAAGGA(A/G)GACAAGAC-3'
EF2	5'-GGA(G/A)GTACCAGT(G/C)ATCATGTT-3'

PCR components

The elongation factor PCR can be performed using either individual PCR ingredients or a commercial PCR mastermix that has been validated by the laboratory. A typical reaction volume is 20 or 25 µL, and should contain 0.5U Taq, 1.5mM $MgCl_2$, 0.2µM of each primer, and 1 µL of DNA extract. A blank is required, but in this case a positive control is not required, as all extracts should yield a PCR product.

PCR Conditions

- 94ºC for 30 sec (unless enzyme manufacturer recommendations are different)
- 40 cycles of 94ºC for 1 min., 55ºC for 1 min., 72ºC for 2 min.
- Hold at 4ºC

PCR results

PCR product can be visualised on an agarose gel or equivalent using standard laboratory methods. An approximate 700bp product should be amplified from all isolates. These PCR products can be sequenced directly and used in a Blast search at http://isolate.fusariumdb.org to confirm that the isolate is *F. oxysporum*. Failure to amplify the EF region may indicate that the isolate is not a *Fusarium* species and a subsequent morphological examination is therefore recommended to determine if re-extraction of the DNA is required.

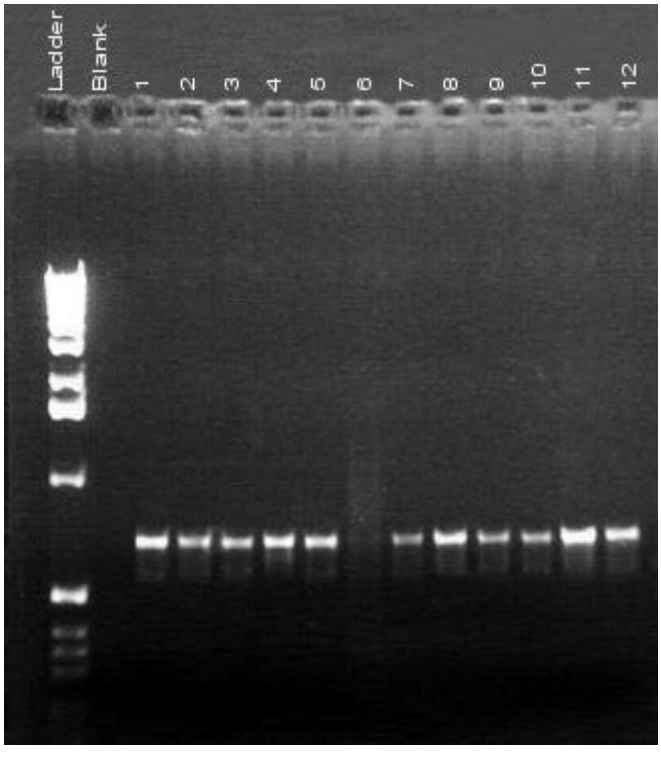

Figure 4. Example gel from DNA evaluation PCR using elongation factor primers on ten randomly selected *Fusarium oxysporum* cultures from herbarium VPRI (lanes 1-10), and two isolates of *Fusarium oxysporum* f.sp. *ciceris* (lanes 11 and 12, details in Figure 5) . Note that the PCR failed for isolate 6 was due to poor DNA quality.

4.3.4 Specific PCR for *Fusarium oxysporum* f.sp. *ciceris*

The DNA extracts successfully amplified by the elongation factor PCR can then be used for the specific *Fusarium oxysporum* f. sp. c*iceris* PCR.

Primers

Table 3. Primer sequences for the detection of fusarium wilt of chickpea (*Fusarium oxysporum* f. sp. *ciceris*) (Jiménez-Gasco and Jiménez-Díaz 2003).

Primer	Sequence
Foc0-12f	5'-GGCGTTTCGCAGCCTTACAATGAAG-3'
Foc0-12r	5'-GACTCCTTTTTCCCGAGGTAGGTCAGAT-3'

PCR components

The *Fusarium oxysporum* f. sp. c*iceris* PCR can be performed using either individual PCR ingredients or a commercial PCR mastermix that have been validated by the laboratory. A typical reaction volume is 20 or 25 µL, and should contain 0.5U Taq, 1.5mM $MgCl_2$, 0.2µM of each primer, and 1 µL of DNA extract. A blank and a positive control is required.

PCR Conditions

- 94°C for 2 min (unless enzyme manufacturer recommendations are different)
- 28 cycles at 94°C for 30 sec., 58°C for 1 min. and 72°C for 30 sec and final elongation 72°C for 4 min.
- Hold at 4ºC

PCR results

PCR product can be visualised on an agarose gel or equivalent using standard laboratory methods. A PCR product of approximately 1500 bp indicates *F. oxysporum* f. sp. *ciceris* (see Figure).

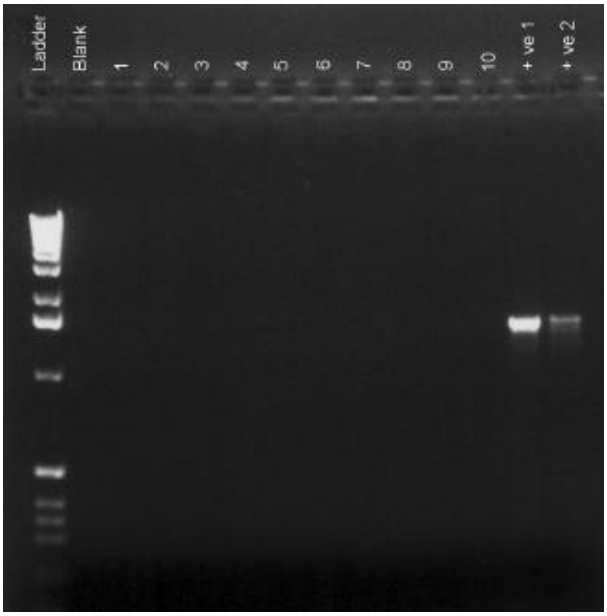

Figure 5. Example electrophoresis gel showing the Blank, *F. oxysporum* cultures 1-10 (from Figure 4), *F. oxysporum* f. sp. *ciceris* Race 1A (+ve 1) and *F. oxysporum* f. sp. *ciceris* Race 5 (+ve 2). Positive control extracts supplied by Rafael M. Jiménez-Díaz, Professor of Plant Pathology, University of Córdoba.

5 CONTACTS FOR FURTHER INFORMATION

Dr James Cunnington
Department of Agriculture & Water Resources
Street Address: 7 London Circuit, Canberra City ACT 2601
Postal Address: GPO Box 858, Canberra City ACT 2601
James.Cunnington@agriculture.gov.au

Dr Kurt Lindbeck
NSW Department of Primary Industries
Wagga Wagga Agricultural Institute
Pine Gully Road
Wagga Wagga
New South Wales 2650
Australia

5.1 Experts who can be contacted for professional diagnostic and advisory services

Expert	State	Details
Joop van Leur	NSW	NSW Department of Primary Industries Tamworth Centre for Crop Improvement 4 Marsden Park Road Calala NSW 2340 Ph: (02) 6763 1100; Fax: (02) 6763 1222
Edward Liew	NSW	Royal Botanic Gardens - Sydney Mrs Macquarie Road Sydney NSW 2000 Ph: (02) 9231 8113; Fax: (02) 9241 1135

6 ACKNOWLEDGEMENTS

The information in this diagnostic protocol was sourced from the National Diagnostic Protocol for the detection of Fusarium Wilt of Chickpea (*Fusarium oxysporum* f. sp. *ciceris*) produced by the Department of Primary Industries, Victoria, and authored by Dr James Cunnington, Dr Kurt Lindbeck and Dr Rodney H. Jones.

We are grateful to Rafael M. Jiménez-Díaz, Professor of Plant Pathology, University of Córdoba, for DNA extracts *of Fusarium oxysporum* f. sp. *ciceris.*

The protocol was reviewed and verified by Dr Jeyaseelan Baskarathevan, Ministry for Primary Industries, New Zealand.

7 REFERENCES

Haware MP, Nene YL (1982) Symptomless carriers of the chickpea wilt Fusarium. *Plant Disease* **66**, 250-251.

Haware MP, Nene YL, Mathur SB (1986) Seed-borne diseases of chickpea. Technical Bulletin, Danish Government Institute of Seed Pathology for Developing Countries.

Jiménez-Gasco MM, Jiménez-Díaz RM (2003) Development of a specific Polymerase Chain Reaction-based assay for the identification of *Fusarium oxysporum* f. sp. *ciceris* and its pathogenic races 0, 1A, 5 and 6. *Phytopathology* **93**, 200-209.

Jiménez-Gasco MM, Milgroom MG, Jiménez-Díaz RM (2002) Gene genealogies support *F. oxysporum* f. sp. *ciceris* as a monophyletic group. *Plant Pathology* **51**, 72-77.

Leslie JF, Summerell BA (2006) The Fusarium Laboratory Manual. Blackwell Publishing Asia, Australia.

Nene YL, Haware MP, Reddy MV (1978) Diagnosis of some wilt-like disorders of chickpea *(Cicer arietinum)*. ICRISAT Information Bulletin No. 3.

Nene YL, Sheila VK, Sharma SB (1996) A world list of chickpea and pigeonpea pathogens. ICRISAT, India.

O'Donnell K, Kistler HC, Gigelnik E, Ploetz RC (1998) Multiple evolutionary origins of the fungus causing Panama disease of banana: concordant evidence from nuclear and mitochondrial gene genealogies. *Proceedings of the Natural Academy of Sciences* **95**, 2044-2049.

Richardson MJ (1990) An annotated list of seed-borne diseases (4th ed.). ISTA Zurich, Switzerland.

Trapero-Casas A, Jiménez-Díaz RM (1985) Fungal wilt and root rot diseases of Chickpea in Southern Spain. *Phytopathology* **75**, 1146-1151.

Singh G, Chen W, Rubiales D, Moore K, Sharma YR, Gan Y (2007) Diseases and their management. In 'Chickpea Breeding and Management'. (Eds SS Yadav, RR Redden, W Chen, B Sharma) pp. 497-519. (CAB International).

8 APPENDIX

8.1 Verification of molecular identification method

8.1.1 DNA extraction

Genomic DNA was extracted from *F. oxysporum* f. sp*. ciceris* cultures according to SPHD protocol (Version V1, section 4.4.1.3) using DNeasy® Plant Mini Kit (Qiagen) and stored at -80°C until used for PCR tests.

8.1.2 Molecular identification method – Evaluation of DNA quality

The quality of the genomic DNA extracted from *F. oxysporum* f. sp*. ciceris* cultures was evaluated according to the draft SPHD protocol (Version V1, section 4.4.1.4) using elongation factor primers EF1 and EF2 (O'Donnell *et al*., 1998). The PCR test was performed with Platinum *Taq* polymerase (Invitrogen) following the PCR reaction and cycling conditions given in the protocol. No amplification was obtained for either DNA extracts. However, amplification was successful after increasing the concentrations of both EF1 and EF2 primers (0.2 µM final concentration) and decreasing the concentration of dNTPs (200 µM final concentration) (Figure 6a). For comparison, this PCR assay was repeated with a pre-mixed master mix, GoTaq Green master mix (Promega), with the PCR reaction and cycling conditions given below.

PCR reaction (20 µl):
10 µl Go *Taq* Master Mix (2x)
1.0 µl EF1 primer (5 µM)
1.0 µl EF2 primer (5 µM)
7.0 µl sterile water
1 µl DNA template (20ng)

Cycling conditions:
94°C for 4 min
94°C for 40 sec
52°C for 1 min } x 35 cycles
72°C for 1 min
72°C 7 min

Following the above reaction and PCR conditions, good amplification was obtained (Figure 6b). Thus, it is advantageous to include alternative PCR cocktails, like the GoTaq Green Master Mix in the protocol since it reduces the number of steps in PCR setup and gel electrophoresis.

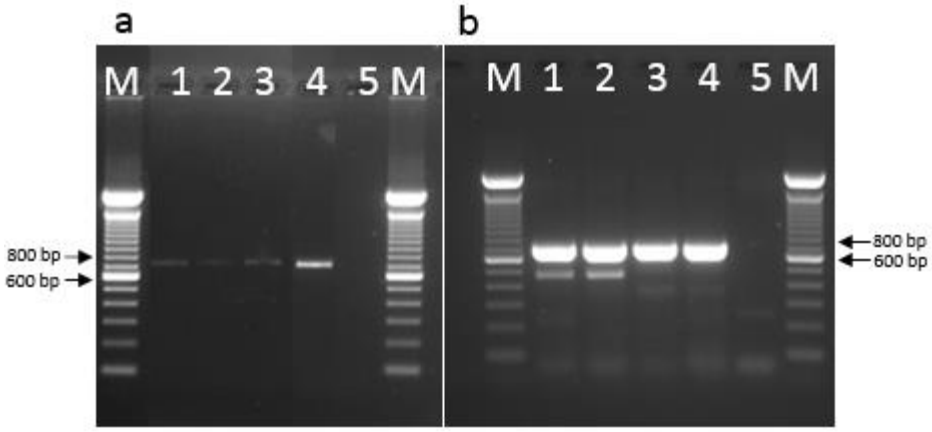

Figure 6. Results from PCR amplification of the elongation factor for DNA quality evaluation with primers EF1 and EF2 using (a) Platinum *Taq* polymerase (modified reaction conditions as described above), and (b) GoTaq Green master mix. Lane numbers indicate *Fusarium oxysporum* f. sp. *ciceris* isolates, IMI 241424 (1 and 2), IMI 259236 (3 and 4), no- template control (5), and M – 100 bp marker.

8.1.3 PCR protocol for Fusarium oxysporum f. sp. ciceris

The PCR method described in section 4.4.2 (Version V1) was tested with Platinum *Taq* polymerase (Invitrogen) according to the PCR reaction and cycling conditions given in the protocol. After several attempts, no amplification was obtained for neither of the *F. oxysporum* f. sp. *ciceris* isolates. The PCR reaction described in the SPHD protocol uses a very low primer concentration (0.008 µM) and a high concentration of dNTP mix (800 µM) when compared to the original publication of this PCR test where the concentrations are 0.2 µM and 200 µM, respectively (Jiménez-Gasco & Jiménez-Díaz, 2003). The PCR test was thus repeated with the primer and dNTP mix concentrations according to the original publication (Jiménez-Gasco & Jiménez-Díaz, 2003) which resulted in amplification (Figure 7a).

For comparison, the PCR assay was repeated with a pre-mixed GoTaq Green master mix, the PCR reaction conditions given below, and the thermocycling conditions given in the SPHD protocol.

PCR reaction (20 µl):
10 µl Go *Taq* Master Mix (2x)
1.0 µl Foc0-12f primer (5 µM)
1.0 µl Foc0-12r primer (5 µM)
7.0 µl sterile water
1 µl DNA template (20ng)

The GoTaq Green master mix gave a good amplification of *F. oxysporum* f. sp. *ciceris* IMI 259236 (Figure 7b). However, the isolate IMI 241424 did not amplify in either PCR tests. To confirm the identity of the isolate IMI 241424, the ITS region and the elongation factor gene were sequenced for both isolates and analysed using Geneious software. The pairwise alignment of the ITS and elongation factor sequences from these two isolates showed 92.27% and 95.05% identity, respectively ~~(Appendix~~

~~1~~). The NCBI BLASTn analysis of the ITS sequence of IMI 241424 showed 100% identity to *F. acutatum* (Accession U34573) and *Fusarium proliferatum* (Accession KF222568). The ITS sequence of isolate IMI 259236 showed 100% identity to many *F. oxysporum* isolates sequences previously deposited in the GenBank. Thus, the ITS sequence is not reliable to confirm sub-species identity of *F. oxysporum* f. sp. *ciceris*. Nevertheless, the elongation factor sequence of isolate IMI 259236 showed 100% identity to other *F. oxysporum* f. sp. *ciceris* sequences previously deposited in the GenBank, whereas isolate IMI 241424 showed 100% identity to *F. acutatum*. Thus, the isolate IMI 241424 is most likely mis-identified and does not represent *F. oxysporum* f. sp. *ciceris*. This is also likely the reason why no amplification was obtained for the isolate IMI 241424 using the SPHD molecular test for *F. oxysporum* f. sp. *ciceris*.

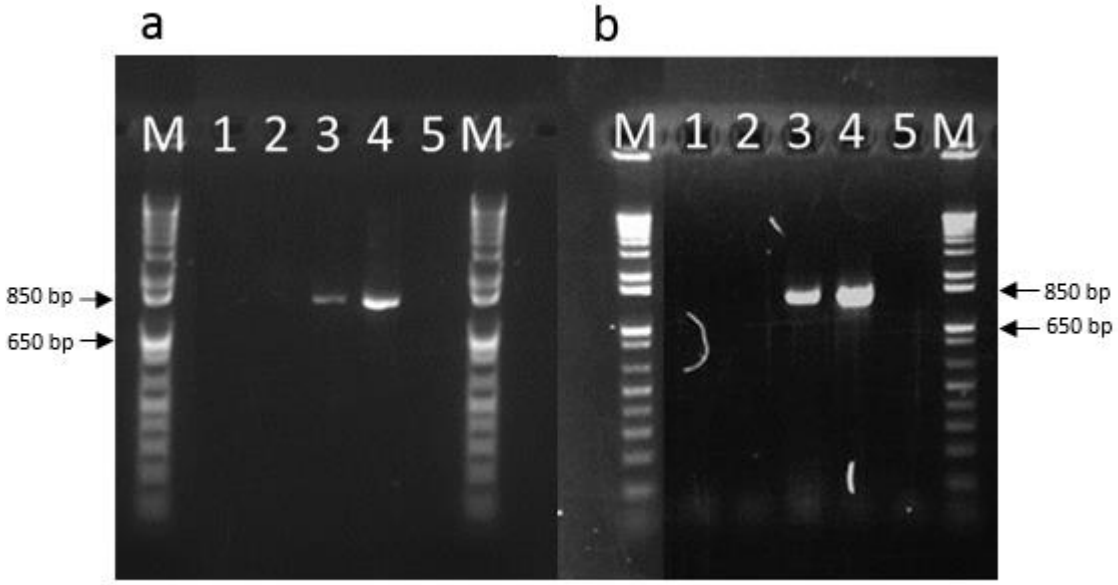

Figure 7. Results from PCR amplification of *Fusarium oxysporum* f. sp. *ciceris* with Foc0-12f and Foc0-12r primers using (a) Platinum *Taq* polymerase and the conditions published in Jiménez-Gasco and Jiménez-Díaz, 2003, and (b) GoTaq Green master mix and conditions described above. Lane numbers indicate *F. oxysporum* f. sp. *ciceris* isolates, IMI 241424 (1 and 2), IMI 259236 (3 and 4), no- template control (5) and M- 1 Kb+ marker.

In conclusion, following the conditions given in the molecular identification section of the SPHD protocol (Version V1), *F. oxysporum* f. sp. *ciceris* could not be identified. However, the molecular test worked after modifying the PCR reaction according to original publication of this PCR test. Also, changing the PCR reaction composition (GoTaq) enabled to obtain better PCR amplifications. Due to a mis-identified culture, this protocol was only tested with a single *F. oxysporum* f. sp. *ciceris* isolate, thus we cannot confirm that the test allows to identify isolates from different geographical origins and races of *F. oxysporum* f. sp. *ciceris*.

8.2 Morphological identification of *Fusarium oxysporum*

Identification of *Fusarium oxysporum* based on morphology can be done with direct visual examination of the pathogen in pure culture. This type of identification is not entirely conclusive and should be done in conjunction with other identification methods. Identification is based on spore size, ornamentation and colour. It will not indicate host specificity. Spores have to be mounted onto slides and inspected using a microscope.

Fusarium oxysporum can defined to some degree by morphological criteria, including the shape of micro- and macroconidia, the structure of the microconidiophore (false heads on short phialides formed on the hyphae), and the formation of chlamydospores. Key characters are observable on selected media (Leslie and Summerell 2006)

Microconidia are abundant, aseptate, reniform to oval, produced in false heads on short monophialide conidiophores. They range from 5-12 µm x 2-3.5 µm. Macroconidia are rare. Chlamydospores are profuse in culture and are formed singly or in pairs.

On Carnation Leaf-piece Agar (CLA), macroconidia of *Fusarium oxysporum* are formed in pale orange sporodochia borne from monophialides on branched conidiophores, or sometimes from monophialides on hyphae. The macroconidia are short to medium in length, falcate to almost straight, thin-walled and usually 3-septate. The basal cell is notched or foot shaped, and the apical cell slightly hooked in some isolates. Microconidia are formed abundantly in false heads on short monophialides (Figure 8). They may be oval, elliptical or reniform, and are usually without septa.

In most isolates, chlamydospores are formed abundantly and rapidly (2-4 weeks), but formation may be slow (4-6 weeks) or absent in some isolates. Chlamydospores are usually formed singly or in pairs, but may be found in clusters or small chains. They may be either terminal or intercalary, and are most obvious in hyphae on the agar surface, although they may appear in submerged hyphae.

On Potato Dextrose Agar (PDA), colony morphology varies widely. Colony growth on PDA is rapid with white aerial mycelium that may become slightly tinted with orange. The undersurface may be colourless to faintly green or blue. Colony colour is largely dependent on incubation conditions. *Fusarium oxysporum* usually produces a pale to dark violet or dark magenta pigment in the agar, but some isolates produce no pigment at all. Mycelia may be floccose; sparse or abundant; and range in colour from white to pale violet. Abundant pale orange or pale violet macroconidia are produced in a central spore mass in some isolates. Small pale brown, blue to blue-black or violet sclerotia may be produced abundantly in some isolates. The appearance of some isolates is influenced by mutation to the pionnotal form or to a flat 'wet' mycelial colony with a yellow to orange appearance on PDA.

Although there is considerable variation in these structures, *F. oxysporum* can be distinguished from *F. solani*, which forms microconidia on false heads on long monophialides, and from *F. subglutinans*, which forms microconidia on polyphialides and does not form chlamydospores.

Figure 8. Microconidia in false heads on short monophialides on hyphae of *Fusarium oxysporum.* (Photo J. Cunnington 2003)

8.3 Identification based on host species and seedling testing

Identification of the *formae speciales* of *F. oxysporum* can be done through pathogenicity testing on a set of differential species. This would indicate the host specificity of the isolate and hence whether the isolate is exotic.

A suggested set of differential plant species would include:

- Lentil (*Lentil culinaris*) – widely grown commercial variety, but not 'Nipper' or 'Northfield' which are resistant
- Field pea (*Pisum sativum*) – variety selection is not important as all are susceptible
- Chickpea (*Cicer arietinum*) – variety selection is not important
- Faba bean (*Vicia faba*) – variety selection is not important
- Vetch (*Vicia sativa*) – variety selection is not important
- Lupin (*Lupinus albus*) – variety selection is not important

A positive test result for *F. oxysporum* f. sp. *ciceris* would be a susceptible reaction on lentil, chickpea and field pea only. Faba bean, vetch and lupin would be resistant.

A seedling test may also be performed on suspected infected seed to look for symptoms of the disease. A method is suggested by Haware *et al*. (1986) for the identification of fusarium wilt of chickpea.

Equipment

- Suspected chickpea seed sample
- 2 % Sodium hypochlorite (NaOCl)
- Peat soil or fine river sans
- 10 cm pots

Procedure

- Surface sterilise chickpea seeds for 2 mins in 2 % sodium hypochlorite (NaOCl) solution.
- Sow seeds into 10cm pots filled with either peat soil or fine river sand.
- Maintain pots in growth chambers at 25°C in an alternate cycle of 12 h light and 12 h darkness.
- Plants should be observed for 40 days for symptoms of wilt.
- Infected seedlings generally show wilt symptoms between 15 and 25 days after sowing. Infected seedlings are generally pale in colour and show drooping of the leaves. Roots do not show any external rotting, however the fungus can be isolated from the roots.