National Diagnostic Protocol for the detection of Fusarium Wilt of Chickpea (*Fusarium oxysporum* f. sp. *ciceris*)

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Disclaimer

CONTENTS

List of Tables

List of Figures

List of Appendices

1. Introduction

Within the *Fusarium* genus, *F. oxysporum* is without doubt the most economically important species (Leslie and Summerell 2006). The anamorphic soil-borne fungus *Fusarium oxysporum* f. sp. *ciceris* (Padwick) Matuo & K.Satô is pathogenically associated with *Cicer* spp., of which the high-value pulse crop, chickpea (*Cicer arietinum* L.) is the only cultivated species. Worldwide distribution of chickpea production for 2004 was dominated by India (c. 70%), with the countries of Turkey, Pakistan, Iran, Mexico, Myanmar, Ethiopia and Australia making up the other major producers respectively. Other significant production areas include South Asia, the Middle East and Northern Africa, as well as Southern Europe, Canada and the USA.

In Australia, chickpea production is mainly devoted to export as human food. From commencement of commercial cropping in the early 1980s, chickpea is now established as an important crop in northern farming systems of NSW and southern Queensland. These two States contribute the majority of overall production, with Victoria, Western Australia and South Australia making minor contributions to the area under production. NSW and Queensland produce the majority of Desi chickpeas, while Victoria produces the majority of Kabuli chickpeas.

Several diseases are known to limit worldwide production of chickpeas, of which *Fusarium oxysporum* f. sp. *ciceris* (fusarium wilt) is one of the most important. Management of fusarium wilt has been primarily through development of resistant cultivars as part of an integrated management approach. However, the high pathogenic variability in populations of *F. oxysporum* f. sp. *ciceris* presents problems for sustainability of resistant cultivars.

Two pathotypes and eight races of the pathogen have been identified. The reliance on resistant cultivars for disease management of fusarium wilt therefore places significant importance on the confident and efficient identification of pathogenic races of *F. oxysporum* f. sp. *ciceris*. Using non-molecular methods, determination of the organism to the taxonomic level of *formae specialis* is costly in terms of time and resources. A PCR-based molecular assay has been developed that addresses these issues (Jiménez-Gasco and Jiménez-Díaz 2003).

Although other *formae speciales* of *Fusarium oxysporum* are associated with plant groups in Australia, including important crops such as banana and cotton, *F. oxysporum* f. sp. *ciceris* is not known to occur in Australia. This publication documents a molecular test for the identification of all races of *F. oxysporum* f. sp. *ciceris* in the event that the pathogen is introduced to Australia.

2.0 Pest Risk Analysis

2.1 Background

Fusarium wilt of chickpea (*Fusarium oxysporum* f. sp. *ciceris*) is an exotic disease to Australia.

2.2 Species name

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

2.3 Synonyms

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

2.4 Common names

Fusarium wilt of chickpea
Wilt of chickpea
Wilt of pigeonpea

2.5 Host range of *Fusarium oxysporum* f. sp. *ciceris*

Table 1. Host range of *Fusarium oxysporum* f. sp. *ciceris*, with associated reference.

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)

2.6 Distribution

2.6.1 *Australian status*

Fusarium oxysporum f. sp. *ciceris* is not known to occur in Australia, and is considered exotic. The disease was not found in Victorian chickpea production areas surveyed in 2005 (unpublished data).

2.6.2 *Current distribution*

Nene and Reddy (1987) record a distribution of *F. oxysporum* f. sp. *ciceris* covering North America, Europe, Middle East, Asia and South East Asia.

Global distribution is correlated to the presence of designated races of *F. oxysporum* f. sp. *ciceris*, following Jiménez-Gasco and Jiménez-Díaz (2003):

- India – races 2, 3 and 4
- India, Mediterranean and USA (California) – race 1A
- Mediterranean and USA (California) – races 0, 1B/C, 5 and 6

2.6.3 *Potential distribution in Australia*

The potential distribution of fusarium wilt of chickpea is considered to be those regions where the main host crop chickpea is grown (Fig. 1). In Australia this includes central and southern Queensland, the northern and southern cropping belts of New South Wales, the Wimmera and Mallee regions of Victoria, the Yorke and Eyre Peninsulas of South Australia, and areas of the Western Australian cropping belt. However, other leguminous crop species including field pea and lentil can also host *Fusarium oxysporum* f. sp. *ciceris,* and are grown in similar regions throughout the Australian grain belt.

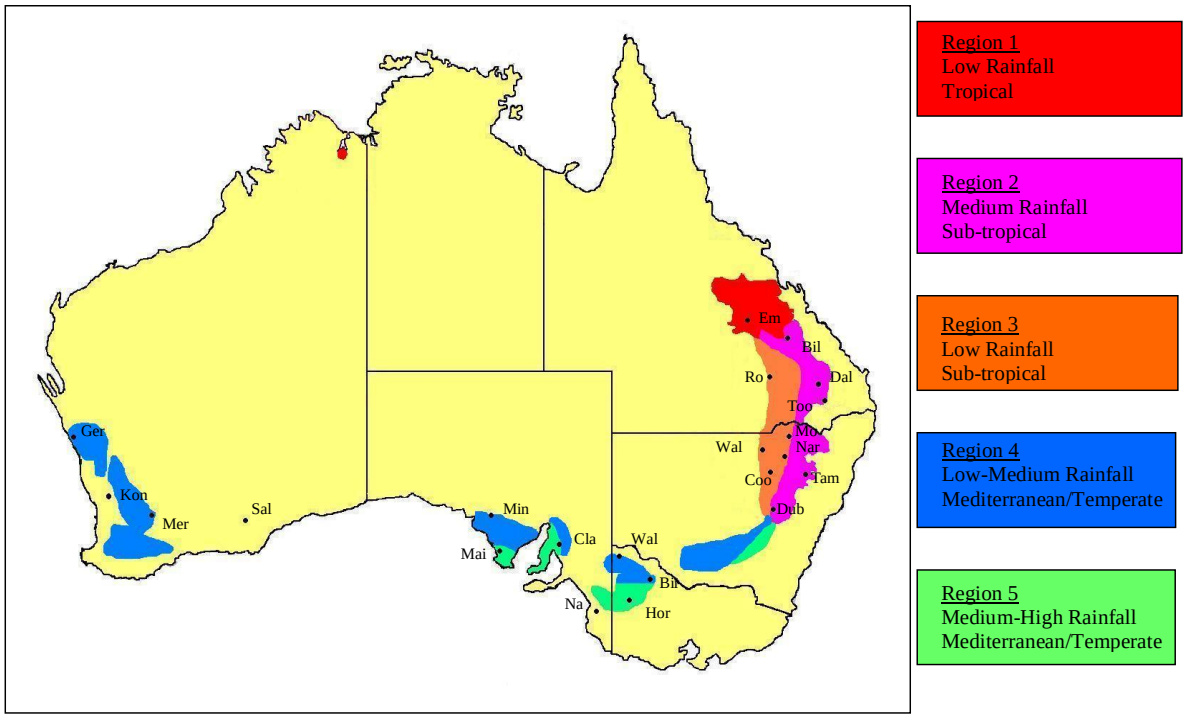

Fig. 1. Distribution of the main eco-geographic chickpea production areas in Australia, reflecting the potential distribution of fusarium wilt of chickpea.

2.7 Plant parts affected

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

2.7.1 Seedborne Hosts

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

2.8 Other root diseases of chickpea

There are several common root diseases of chickpea in Australia; these include phoma blight (*Phoma medicaginis* var*. pinodella*), damping off (*Pythium* spp.), rhizoctonia root rot (*Rhizoctonia* spp.) and phytophthora root rot (*Phytophthora medicaginis*). Fusarium wilt of chickpea may be easily mistaken for one of these common root diseases, but there are several key symptoms that are unique to this disease. Section 2.9 outlines some important aspects of the disease.

2.9 Important aspects of the disease

2.9.1 *Key characters observable on selected media* (Leslie and Summerell 2006)

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.

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 (Fig. 2). They may be oval, elliptical or reniform, and are usually without septa (Fig. 3).

Fig. 2. Microconidia in false heads on short monophialides on hyphae of *Fusarium oxysporum*.

Fig. 3. Microconidia of *Fusarium oxysporum*.

In most isolates, chlamydospores are formed abundantly and rapidly (2–4 weeks), but formation may be slow (4–6 weeks) or not at all 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. *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.

Identification to infraspecific level for *F. oxysporum* using morphology is problematic due to the diversity of non-pathogenic or saprophytic strains in soil, and the difficulties in distinguishing these from pathogenic strains based on morphological or cultural criteria. The inherent cost in terms of time and resources to characterise isolates to *Fusarium oxysporum* f. sp. *ciceris*, and further into pathogenic races, has necessitated the development of molecular methods to achieve satisfactory determinations. A PCR-based assay has been developed (Jiménez-Gasco and Jiménez-Díaz 2003) that will identify all races of the pathogen. The assay involves isolation the organism from infected plant material, DNA extraction and amplification using a specific polymerase chain reaction (PCR).

2.9.2 *Taxonomic groupings within* Fusarium oxysporum

Fusarium oxysporum Schltdl. is one of the most variable and highly dispersed species of *Fusarium*. The variability of *F. oxysporum* is reflected in the distribution and ecological activities of the species. Substantial populations are found in many native plant communities as well as in areas under cultivation, where they may be aggressive colonisers of the root cortex but are predominately non-pathogenic. Research activity has focused on the pathogenic strains occurring in agricultural soils.

The high level of host specificity of pathogenic strains in *F. oxysporum* led to the development of the *formae speciales* concept to enable better differentiation of these morphologically similar strains. Although host range is usually restricted to a few plant species, some *formae speciales* may have broader host ranges. As such, these groupings generally reflect phenotypic characteristics, and are not necessarily indicators of genetic relatedness, with one possible exception being *F. oxysporum* f. sp. *ciceris* (Jiménez-Gasco *et al.* 2002).

Vegetative compatibility, where two hyphae anastomose to form a stable heterokaryon, has been used to genetically distinguish and classify strains of *F. oxysporum*. However, genetic uniformity is not guaranteed for strains belonging to the same vegetative compatibility group (VCG) (Leslie and Summerell 2006). The implication here is that some VCGs may contain both pathogenic and non-pathogenic strains towards a common host.

Symptom type (see Section 2.9.3 Symptoms) has been used to subdivide *F. oxysporum* f. sp. *ciceris* into two pathotypes (Trapero-Casas & Jiménez-Díaz 1985), designated wilting pathotype and yellowing pathotype. Pathotypes are assigned to pathogenic races according to variation in virulence. Races can be defined by differential disease reaction on chickpea host genotypes. For *F. oxysporum* f. sp. *ciceris*, eight races with distinct geographic distributions (see Section 2.6.2 Current Distribution) have been identified.

2.9.3 *Symptoms*

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 4 shows typical distribution of chickpea plants infected with *F. oxysporum* f. sp. *ciceris* under field conditions. Careful examination of infected roots (Fig. 5) can differentiate fusarium wilt from other diseases of seedlings and roots of chickpea. Wilt can be observed within 25 days of sowing 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.

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 2 to 3 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 a yellowing and drying of the lower leaves, and a stunting of the plant. Roots will show internal discolouration. Figure 6 shows typical yellowing symptoms.

Fig. 4. Typical distribution of chickpea plants infected by *Fusarium oxysporum* f. sp. *ciceris* under field conditions. (Photo taken in Syria 2002).

Fig. 5. Cross section of a chickpea tap-root showing internal discoloration caused by *Fusarium oxysporum* f. sp. *ciceris* infection.

Fig. 6. An uprooted chickpea plant affected by fusarium wilt, clearly showing 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 faster than plants originating from clean seed.

2.9.4 *Disease cycle*

Following infection of host roots, the fungus crosses the cortex and enters the xylem tissues. It then spreads rapidly up through the vascular system, becoming systemic in the host tissues, and may directly infect the seed.

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. *F. 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 6 years (Singh *et al*. 2007).

2.9.5 *Dispersal*

Dispersal of *Fusarium oxysporum* f. sp*. ciceris* can occur in several forms, these being infested plant debris (root, leaf and stem), soil and seed. Each can be important in the spread and establishment of the disease:

- The principal means of dispersal of the pathogen over short distances is by water or contaminated farm equipment. Conidia can be dispersed by water flow, rainsplash and by movement of infected soil or plant material.
- Over longer distances the pathogen may be dispersed in infected plant debris, seed and chlamydospores in associated soil.
- Once in an area, *F. oxysporum* f. sp*. ciceris* survives between crops in infected plant debris as mycelium, microconidia, macroconidia and, most commonly, as chlamydospores. The pathogen is able to survive for many years either in soil as chlamydospores or as a saprobe in plant debris.

2.10 Assessment of likelihood of introduction into Australia

The following risk analysis is based on the methodology in Biosecurity Australia's guidelines on Import Risk Analysis (2001).

2.10.1 *Entry potential*

Entry potential is **Low**, but possible given the following factors:

- Australia currently imports 150 – 200 tonnes of chickpeas for human consumption annually. Current AQIS import conditions require that imported consignments be accompanied by a phytosanitary certificate. Despite this legislation, there is no guarantee that the pathogen cannot enter via infected seed or infested lentil trash that may accompany the consignment.

2.10.2 *Host range potential*

Host range potential is **Medium** as *Fusarium oxysporum* f. sp. *ciceris* has a moderately complex host range involving a number of plant families.

2.10.3 *Establishment potential*

Establishment potential is considered to be **High**, as:

- *Fusarium oxysporum* already occurs in Australia on other crop host species, demonstrating that suitable conditions do occur in Australia for the pathogen to survive;
- climatic conditions between countries such as Syria where the disease already occurs and areas of Australia are similar;
- chlamydospores of the pathogen can survive in the soil for many years in the absence of a host plant. The pathogen can also survive within infected plant material in the field;
- current commercial chickpea cultivars in Australia are highly susceptible to chickpea fusarium wilt; and
- *Fusarium oxysporum* f. sp. *ciceris* can also be hosted by lentil and field pea which are widely grown throughout the Australian cropping belt.

2.10.4 *Spread potential*

Spread potential is considered to be **High**, given the following:

- spores can be splash dispersed, rain splash and moving water can carry chlamydospores and conidia short distances to surrounding plants and adjoining paddocks;
- the pathogen can be transported over large distances in infected grain and harvesting equipment into new areas;
- grain infected by *Fusarium oxysporum* f. sp. *ciceris* may not show external symptoms of infection; and
- wind blown plant debris could spread the pathogen over moderate distances following harvest into adjacent paddocks.

2.11 Overall entry, host range, establishment and spread potential

Low – The probability of entry, establishment and spread is determined by combining the likelihoods of entry, host range, establishment and spread.

2.12 Assessment of consequences of entry into Australia

2.12.1 *Economic impact*

High – This disease has the potential to greatly downsize the chickpea industry in Australia in a similar manner to the ascochyta blight outbreak in 1998. An outbreak of fusarium wilt of chickpea would result in a dramatic reduction in the area of production, due to increased costs of production making chickpea less competitive compared to other crops.

A substantial loss would also be incurred in the year of the outbreak. This not only includes lost production but also indirect impact on other business sectors such as other agricultural enterprises, storage, transport, manufacturing and wholesale trade. The losses would be similar to those incurred as a result of the outbreak of ascochyta blight in chickpeas in 1998 which has been calculated to have cost the Wimmera region in Victoria $62 million.

This disease causes serious yield losses in those countries where the pathogen is known to occur. Yield losses of up to 60% may occur under favourable conditions (Singh *et al*. 2007).

2.12.2 *Environmental impact*

Negligible – There is no potential to degrade the environment or otherwise alter the ecosystem by affecting species composition or reducing the longevity or competitiveness of wild hosts.

2.12.3 *Social impact*

Moderate – The reduction in the value of production would be expected to cause moderate social impact with significant losses to local and broader communities.

2.13 Combination of likelihood and consequences to assess risks

- Economic risk: **High** – specific action is immediately required to reduce risk.
- Environmental risk: **Low** – manage through routine procedures.
- Social risk: **High** – adoption of generic risk treatment plans will reduce the risk to appropriate levels.

2.14 Surveillance

Maintaining vigilance for exotic pathogens within the grains industry is clearly important. Recent incursions of lupin anthracnose and chickpea ascochyta blight in Australia may have been avoided had industry been aware of exotic grains threats and their identification. Chickpea growers and field crop agronomists should be encouraged to report any unusual symptoms to their State Departments of Agriculture where an experienced plant pathologist can perform identification. If effective control and possible containment of an exotic plant disease is to occur then rapid identification of the disease is necessary.

2.15 Diagnostics

Diagnosis of fusarium wilt of chickpea is a two-stage process. Firstly, a preliminary microscopic examination is undertaken to determine whether disease symptoms and pathogen morphology are consistent with chickpea fusarium wilt or an endemic disease, such as phoma or rhizoctonia root rot or phytophthora root rot. The primary diagnostic test, a PCR, is undertaken to determine if the pathogen is *F. oxysporum* f. sp. *ciceris*. An experienced plant pathologist should perform the preliminary examination. The primary test requires sample processing in a specialised laboratory capable of molecular techniques.

The diagnosis of fusarium wilt of chickpea would need to be performed quickly and accurately. The accompanying report describes methods of diagnosing fusarium wilt of chickpea using conventional PCR methods.

2.16 Training

Due to the similarity of fusarium wilt of chickpea symptoms to those of other diseases affecting chickpea, such as phoma or phytophthora root rot, growers and field agronomists need to be aware of the importance of this disease. While it is not possible for all personnel in the field to be trained in disease symptoms and identification, a general awareness of crop disorders and an awareness of availability of local diagnosticians are critical for early disease detection and hence industry protection.

3.0 Isolation of organism and molecular verification

3.1.1 *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)

3.1.2 *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 NaOCl for c. 1 min., 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
- store at 20ºC until adequate fungal growth is observed

3.2 Polymerase Chain Reaction (PCR)

3.2.1 *Laboratory requirements*

- protective gloves
- 2.0, 20 and 300 µL pipettes and sterile plugged tips
- microcentrifuge and microcentrifuge tubes (1.5 mL)
- 0.2 mL PCR tubes
- thermocycler
- gel tray with suitable comb/s, electrophoresis tank and powerpack
- UV transilluminator
- camera/gel documentation system

3.2.2 *Components*

- DNeasy® Plant Mini Kit (Qiagen)
- PCR reagents (see Tables 2, 3 and 4)
- running buffer (e.g. 0.5× TBE, see Appendix 1)
- agarose
- 10 mg/mL ethidium bromide (see Safety Note)
- DNA Ladder
- gel loading dye

3.2.3 *Extraction of DNA*

Clean subcultures from fungal cultures established as in Section 3.1.2 are grown on PDAA until there is adequate material for DNA extraction using a DNeasy® Plant Mini Kit (Qiagen) as per the manufacturer's instructions.

3.2.4 *Quality testing of DNA*

A PCR using elongation factor primers EF1 and EF2 (Table 2) is undertaken to evaluate the quality of DNA extracted from plant material. Other non-primer reagents and cycles used in the evaluation PCR are set out in Section 3.2.5. Samples should produce a PCR product of approximately 560 bp (see Fig. 7).

Table 2. Primer sequences used in PCR to evaluate quality of 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'

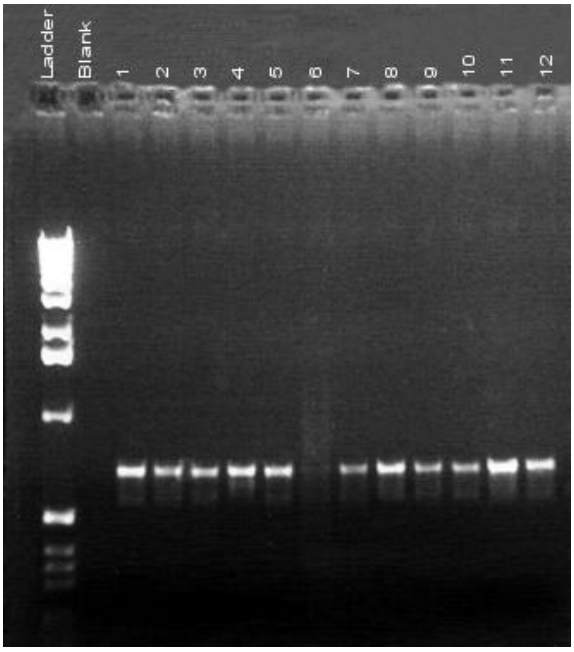

Fig. 7. Example gel from DNA evaluation PCR using elongation factor primers. Results similar to Lane 6 indicate that DNA extraction needs to be repeated for that sample.

3.2.5 *PCR protocol*

1. Prepare a reaction mix as described below (Tables 3, 4) for the number of test samples, a blank and one extra. PCRs should be set up at a dedicated workbench away from the area where the DNA was extracted and electrophoresis is done.

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'

Table 4. Volumes of reagents used in PCR reaction for detection of fusarium wilt of chickpea (*Fusarium oxysporum* f. sp. *ciceris*).

Component	Volume (µL)
Nuclease-free water	17.4
10× PCR reaction buffer + Mg	2.5
dNTP mix (10 mM of each dNTP)	2.0
Primer Foc0-12f (2 µM)	1.0
Primer Foc0-12r (2 µM)	1.0
Taq DNA Polymerase (5 Units/ µL)	0.1
Total	24.0

2. Into separate 0.2 mL PCR tubes, add 24 µL of reaction mix and 1 µL of DNA.
3. Place the reaction tubes in a thermal cycler and subject to one cycle at 94°C for 2 min., followed by 28 cycles at 94°C for 30 sec., 58°C for 1 min. and 72°C for 30 sec.
4. Store PCR products at 4°C.

3.2.6 *Electrophoresis procedure*

1. Determine size of gel and gel comb required (see Table 5).

Table 5. Volume of gel solution required for specific gel tray sizes.

Gel tray size (cm)	Volume (mL) of gel solution required (for 0.7 cm thickness)
7.7 × 9.5	50
14.2 × 15.0	150
18.3 × 25.0	300

2. Place gel tray with appropriate comb(s) in tray holding device.
3. Weigh agarose (1.5 g/100 mL of buffer) and place into suitable heat proof container.
4. Add required amount of gel running buffer (0.5× TBE).
5. Heat until agarose is completely dissolved then allow to cool to approximately 50–55ºC.
6. Add required amount of ethidium bromide (final concentration 0.5 µg/mL) to agarose buffer solution and mix gently.

Safety Note: Ethidium bromide is a carcinogen; wear protective gloves and wash hands thoroughly after use.

7. Pour agarose into gel tray and allow to set.
8. Place set gel in electrophoresis tank and submerge with 0.5× TBE to a depth of at least 1 mm above the gel surface.
9. Load 5 µL of DNA Ladder into the first well of the gel.
10. Add 1 µL of 6× loading buffer per 5 µL of PCR product and load into the individual wells of the gel.
11. Connect electrodes to powerpack and apply a constant voltage of 100 Volts. Run gel for a period of time suitable for the size of selected gel. (c. 30–40 min. for 7.7 cm × 9.5 cm gel).
12. View gel on UV transilluminator, and record image using camera or gel documentation system.
13. A PCR product of approximately 1600 bp indicates *F. oxysporum* f. sp. *ciceris* (see Fig. 8).

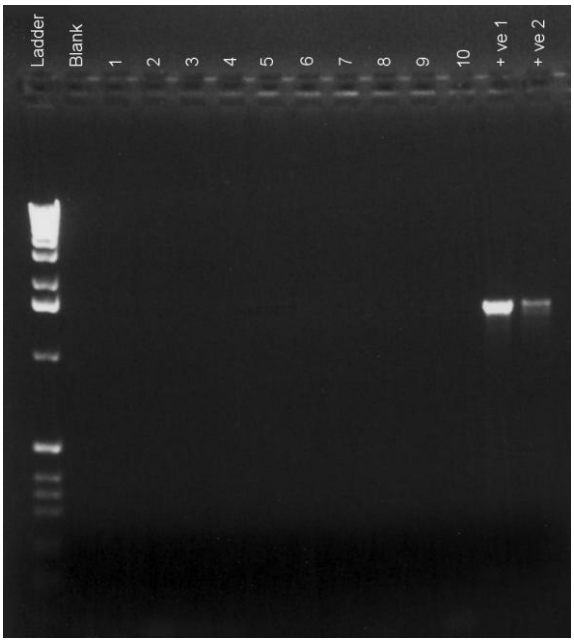

Fig. 8. Example electrophoresis gel showing 100 bp DNA Ladder, empty lanes for the Blank and samples 1-10 as indicated, and bands in lanes corresponding to +ve Control 1 (*F. oxysporum* f. sp. *ciceris* Race 1A) and +ve Control 2 (*F. oxysporum* f. sp. *ciceris* Race 5).

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5.0 Appendices

Appendix 1. Components of TBE (Tris/borate/EDTA) electrophoresis buffer

Component	10× stock solution	5× stock solution
Tris base	108 g	54 g
Boric acid	55 g	27.5 g
0.5 M EDTA pH 8.0	40 mL	20 mL

For TBE, a working solution of 0.5× provides more than enough buffering power, and almost all agarose gel electrophoresis is carried out using this buffer.

Store stock and working TBE solutions at room temperature.

Appendix 2. Gel loading buffers

6× loading buffer (store at 4°C)

0.25% bromophenol blue
0.25% xylene cyanol FF
30% glycerol in water

10× loading buffer (store at 4°C)

0.25% bromophenol blue
0.25% xylene cyanol FF
20% Ficoll 400
M EDTA, pH 8.0
1.0% SDS