

National Diagnostic Protocol

Diaporthe helianthi

(the cause of sunflower stem canker)



NDP 40 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

<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 *Diaporthe helianthi* is current as at the date contained in the version control box below.

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Further information

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1 INTRODUCTION

Stem canker caused by *Diaporthe helianthi* is considered one of the most important diseases of sunflower (*Helianthus annuus*) worldwide (Thompson *et al.* 2011, 2015, Rekab *et al.* 2004, Debaeke *et al.* 2003, SAYS-LESAGE *et al.* 2002, Herr *et al.* 1983). The fungus survives on stubble and under favourable warm, wet conditions infects leaves then grows from the leaf margin up the veins to form cankers at the stem nodes. As the infection develops and compromises the pith behind the nodes the entire plant may wilt (Masirevic and Gulya 1992) and eventually lodge.

Other recent investigations of more than 2000 *Diaporthe* isolates from a range of hosts in Australia have revealed a complex of previously undescribed *Diaporthe* species associated with sunflower and other crops (Thompson *et al.* 2011, 2015) although *D. helianthi* has not been recorded.

To date, as part of this ongoing investigation, eleven new *Diaporthe* species with a range of virulences on sunflower and other hosts have been described by Thompson *et al.* (2011, 2015). The most damaging species on sunflower in Australia is *D. gulyae* although a number of other newly identified species can also cause significant damage under favourable conditions. The results of virulence studies will be published in 2017 (Thompson *et al.* manuscript in progress).

Other systematic studies have determined that several species of *Diaporthe* infect *H. annuus* overseas (Rekab *et al.* 2004, Vergara *et al.* 2004) with *D. helianthi* generally considered the most damaging. However, Matthew *et al.* (2015) have now reported that *D. gulyae* is potentially as damaging as *D. helianthi*.

Morphology is inadequate to distinguish *D. helianthi* from other species of *Diaporthe* on sunflower however species descriptions and photographs of conidia and pycnidia in Thompson *et al.* (2011, 2015) and Santos *et al.* (2011) would be valuable resources.

Molecular barcoding of two loci with comparison to sequences obtained from the type culture of *D. helianthi* is the most effective method for identification.

1.1 Host range

Diaporthe helianthi was described on *H. annuus* in the former Yugoslavia (Muntanola-Cvetkovic *et al.* 1985, Muntanola-Cvetkovic *et al.* 1981). An investigation by Vrandecic *et al.* (2010) concluded *D. helianthi* could cause infection on *Arctium lappa*, *Xanthium italicum*, and *X. strumarium* from the Asteraceae family. Other hosts of *D. helianthi* have been reported, however these identifications have not been validated against the type material (Thompson *et al.* 2011).

2 TAXONOMIC INFORMATION

Classification

Kingdom: Fungi
Phylum: Ascomycota
Sub-phylum: Pezizomycotina
Class: Sordariomycetes
Order: Diaporthales
Family: Valsaceae
Genus: *Diaporthe*
Species: *helianthi* Munt.-Cvetk, Mihljc. & M. Petrov

Synonyms

Phomopsis helianthi Munt.-Cvetk, Mihljc. & M. Petrov

Common names

Sunflower stem canker
Grey stem spot
Phomopsis grey stem

3 DETECTION

Initial infection of *D. helianthi* begins on the lower or middle leaves and spreads via the petioles to the stem where it forms a lesion at the node. Behind the node, pith damage occurs as the fungus invades the tissues and the size of the lesion eventually extends up and down the stem and may also cause girdling.

Advanced infection may cause mid-stem lodging as the heads fill. Lodging may be associated with a lesion but also may not necessarily occur at the point of infection as the lodging is due to a weakened stem collapsing as the weight of the head increases at seed fill.

An easy field test is to firmly press thumb and forefinger against an advanced lesion – the stem will give way due to the pith damage behind the lesion. Smaller lesions may still feel firm to the touch so this pinch test is a guide only.

3.1 Symptoms

Masirevic and Gulya (1992) described the symptom progression of *D. helianthi*. Small necrotic spots surrounded by a chlorotic border develop on the leaf margins and spread down the main veins of a leaf (Fig 1). The infected leaves wilt and die. The fungus spreads from the petiole to the stem, where small, brown, sunken spots develop to large round or ellipsoid lesions that can encircle the stem (Figs 2-4). Destruction of stem pith tissue underneath the lesion leads to wilting and death of the plant (Fig 5) (Masirevic and Gulya, 1992).



Figure 1. Leaf infection, Argentina. Image by Sue Thompson.



Figures 2 & 3. Stem lesion, Argentina. Images by Sue Thompson.



Figure 4. Stem lesion, Argentina. Image by Sue Thompson.



Figure 5. Midstem lodging, Argentina. Image by Sue Thompson.

3.2 *Diaporthe* species causing similar symptoms

Several species of *Diaporthe* are associated with stem cankers on sunflower in Australia. Three of these species were described by Thompson *et al.* (2011), namely *D. gulyae*, *D. kongii* and *D. kochmanii* (the latter has been synonymised as *D. sojiae* by Udayanga *et al.* 2015). *Diaporthe gulyae* produces similar symptoms to those of *D. helianthi* although lesions are generally a brown black rather than the lighter brown of *D. helianthi*. Other species of *Diaporthe* that occur on sunflower internationally have been recognised as a polyphyletic complex (Rekab *et al.* 2004, Says-Lesage *et al.* 2002, Thompson *et al.* 2011, Vergara *et al.* 2004). Recently described new species, such as *D. masirevicii*, *D. miriciae*, and *D. novem* are regarded as milder pathogens on sunflower (Thompson *et al.* 2015). The symptoms and morphological characteristics of these sunflower canker diseases may be similar to *D. helianthi* but virulence levels and lesion types can vary widely.

4 IDENTIFICATION

Morphological identification cannot be reliably used to distinguish *D. helianthi* from other sunflower canker diseases. However, the ex-type strain of *D. helianthi* was observed to readily produce conidiomata (pycnidia) containing mainly β -conidia (Muntanola-Cvetkovic *et al.* 1985). They produced perithecia in the field, but were rarely observed to produce perithecia in culture (Muntanola-Cvetkovic *et al.* 1985). These two cultural traits and minor differences in morphology do not reliably distinguish between isolates of the *D. helianthi* complex. Molecular diagnostic tools are the only reliable methods of identification for these fungi.

4.1 Morphological characteristics

Morphological examination of conidia and asci can determine whether the suspect pathogen belongs to the genus *Diaporthe* (see section 4.1.2).

4.1.1 Isolation of pathogen into culture

Excise small stem pieces from the leading edge of lesions that display brown to brownish-black symptoms. Sterilise the tissue by dipping into 90% ethanol and flaming briefly before placement on 1.5% water agar.

Alternatively, if pycnidia are present in the lesion, place surface sterilised pieces of infected plant tissue onto water agar and incubate for 1-7 d at ambient temperature (21–25°C), then streak out the conidia oozing from a number of single pycnidia onto Potato Dextrose Agar (PDA) amended with 100 $\mu\text{g}/\text{mL}$ streptomycin sulphate (PDAS). (Use water agar amended with 100 $\mu\text{g}/\text{mL}$ streptomycin sulphate (WAS) if it is difficult to separate single spore isolates).

Select single spore isolates or hyphal tip from more advanced cultures and incubate on PDA to establish pure cultures for 7 d under ambient light at 23–25 °C.

Cultures may require 1–5 weeks incubation for the induction of pycnidial formation.

4.1.2 Microscopic identification of *Diaporthe* sp.

To induce sporulation for morphological identification, the isolates need to be grown on PDA with pieces of sterilised wheat stems placed on the surface and incubated under 12 h near-ultraviolet light / 12 h dark at 25 °C. Mount the fungal structures on glass slides in lactic acid (100 % v/v) for microscopic examination after 28 d of incubation.

The ascomata (perithecia) may be present on host material but are generally not observed in culture. Ascomata are globose, ~400 μm in diam., surrounded by a wall comprised of several layers of thick cells; with a prominent beak 350–700 μm long. Asci are clavate-cylindric, 47–57 \times 7–12 μm , with a refractive ring in the apical wall, 8-spored and sessile. Ascospores are irregularly biseriolate, subelliptical, with rounded ends, 1-septate, 12–15 \times 3–4 μm , hyaline conidiomata (pycnidia) are dark-brown, immersed in pseudostromata, aggregate or solitary, globose, 170–320 μm in diameter. Conidiophores simple, rarely branched, arising from innermost layer of cells lining pycnidial cavity. Conidia of the β -type, filiform, hamate, sigmoidal, curved to straight, 22–32 \times 0.5–1.0 μm , unicellular. α -conidia are rarely seen (Muntanola-Cvetkovic *et al.* 1981).

Cultures and spore morphology are illustrated in Gao et al (2017): Available from: https://www.researchgate.net/Diaporthe-helianthi-LC-6185-A-B-7-d-old-culture-on-PDA-C-Conidiomata-D-F-Con_fig3_317306904 [accessed 28 Feb, 2018].

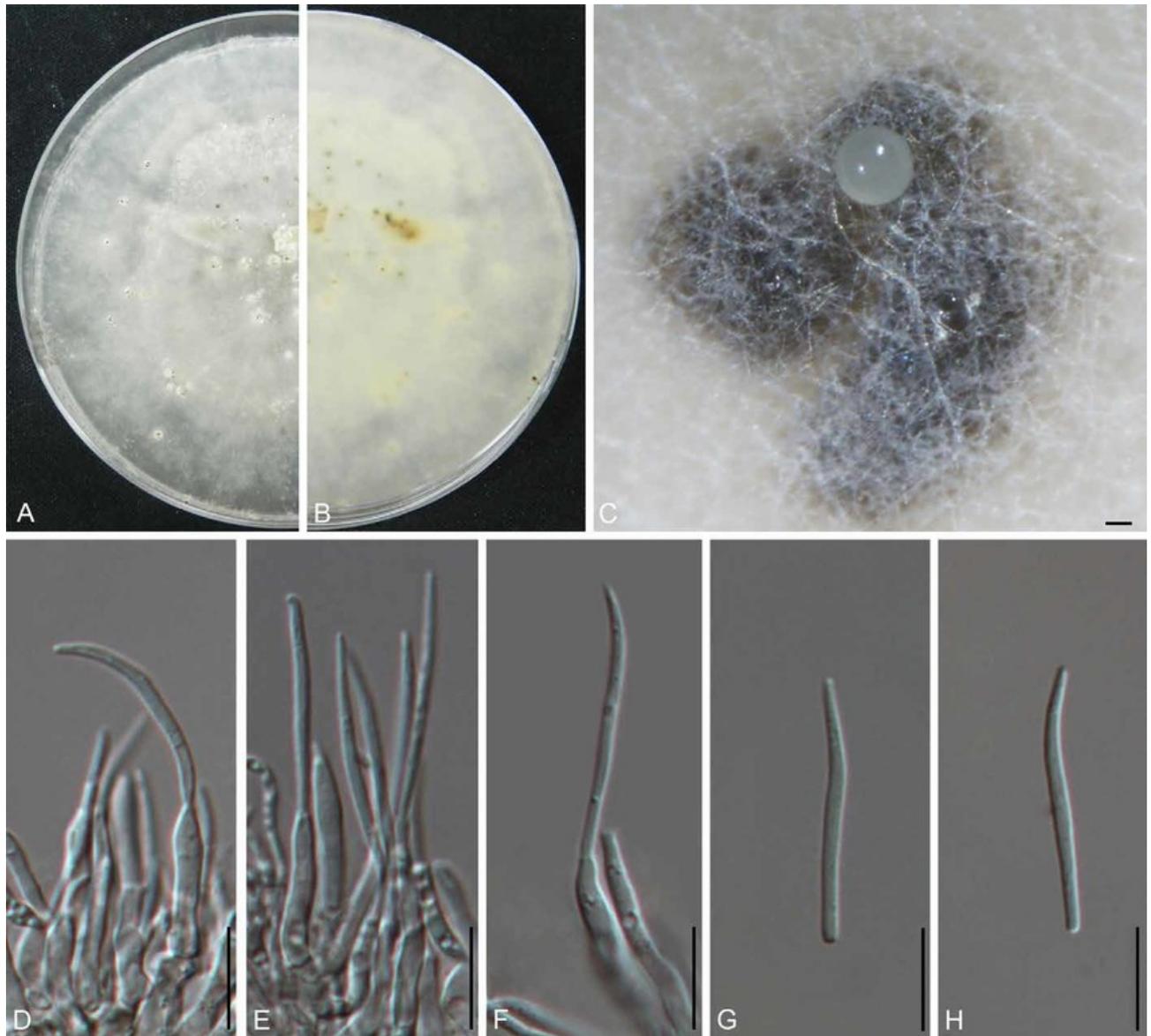


Fig. 6. *Diaporthe helianthi* (LC 6185). A–B. 7-d-old culture on PDA; C. Conidiomata; D–F. Conidiophores; G–H. Beta conidia. Bars: C = 100 µm; D–H = 10 µm. (Gao et al 2017)

4.2 Molecular methods

Diaporthe helianthi can be identified based only on the DNA sequence of the internal transcribed spacer (ITS) region when compared to the ex-type strain. However, to ensure the accuracy of the identification, it is recommended that another gene be sequenced for comparison, namely the β -tubulin (BT) or translation elongation factor 1- α (TEF-1 α) genes.

Equipment

- Disposable gloves (powder free)
- DNA gel electrophoresis apparatus
- Microcentrifuge
- Microcentrifuge tubes (1.5 mL), PCR tubes
- Micropipettes and aerosol resistant tips
- Thermocycler

4.2.1 DNA extraction

Genomic DNA (gDNA) may be extracted from pure fungal cultures using commercially available plant DNA extraction kits; e.g. QIAGEN Plant DNeasy kit, Bioline ISOLATE II Plant DNA extraction kit, etc. The preference is for gDNA to be eluted in nuclease-free H₂O, and store at -20°C until ready to be utilised.

4.2.2 Conventional PCR

Primers and expected product sizes

For amplification and sequencing of the ITS region (de Hoog and van den Ende 1998; White et al. 1990):

V9G (forward)	5'-TTACGTCCTGCCCTTTGTA-3'
ITS4 (reverse)	5'-TCCTCCGCTTATTGATATGC-3'
Expected product size: 540 bp	

For amplification and sequencing of the BT region (Glass and Donaldson 1995; O'Donnell and Cigelnik 1997):

T1 (forward)	5'- AACATGCGTGAGATTGTAAGT-3'
Bt2b (reverse)	5'- ACCCTCAGTGTAGTGACCCTTGGC-3'
Expected product size: 720 bp	

For amplification and sequencing of the TEF-1 α region (Carbone and Kohn 1999; O'Donnell et al. 1998):

EF1-728F (forward):	5'- CATCGAGAAGTTCGAGAAGG -3'
EF2 (reverse):	5'- GGARGTACCAGTSATCATGTT - 3'
Expected product size: 580 bp	

PCR reagents

For DNA sequencing analysis, the preference is to use a high-fidelity PCR master mix to avoid the introduction of PCR errors into the product for DNA sequencing purposes, and to minimise pipetting errors in the preparation of PCR master mix. High-fidelity PCR master mixes can be purchased on a commercial basis, e.g. Phusion® High-Fidelity PCR Master Mix, Platinum® Taq DNA polymerase High Fidelity, etc.

PCR reaction

Reagents	x1	Final conc.
Phusion Master Mix	12.5 µL	1X
Forward Primer (10 mM)	0.5 µL	200 µM
Reverse Primer (10 mM)	0.5 µL	200 µM
Water	10.5	
<u>Template</u>	<u>1 µL</u>	
<u>Total</u>	<u>25 µL</u>	

PCR cycle conditions

The annealing temperatures for a PCR using Phusion® High-Fidelity PCR Master Mix (HF Buffer) on any thermal cycler are as follows:

- ITS and TEF-1α at 55°C;
- BT at 60°C.

Denaturation and extension temperatures and times are in accordance with the manufacturer's protocol.

DNA gel running buffer – TBE Buffer

Tris-Borate-EDTA (TBE) gel running buffer can be purchased commercially in concentrated liquid format. Follow the manufacturer's instructions to dilute it to a 1X concentration. Alternatively, it can be made up from the following components:

	<u>Per 1L</u>	<u>Final conc.</u>
Tris base	54.0 g	0.4 M
Boric acid	27.5 g	0.05 M
0.5M EDTA pH 8.0	20.0 mL	0.001 M

Dissolve components in 1L distilled water. Store at room temperature.

Agarose gel

	Per 100 mL	Conc.
DNA grade agarose	1.0 g	1%
TBE	100 mL	1X

Dissolve the molecular biology-grade agarose in TBE buffer in a heat-proof glass container (e.g. beaker or Schott® bottle) by heating in a microwave. Once the bottle is slightly cool to the touch, pour into the gel tray with comb. It will take approximately 30 mins to set at room temperature (20-22°C).

WARNING: The container and the content are extremely hot. Handle the container with care using heat-proof gloves.

DNA Loading dye and DNA stain

Certain dye powders are hazardous in concentrated forms. Therefore due to the workplace health and safety considerations of dye powders, DNA loading dye should be purchase from commercial companies.

Either Ethidium Bromide or GelRed® may be used to stain double-stranded DNA to visualise it on an agarose gel. The authors recommend to use GelRed® by adding 10 µL of a 10,000X concentration (in water) into 1 mL of DNA loading dye. Vortex well to mix, and store in a dark container when not in use.

Sequence PCR product

Once it is confirmed that there is a single PCR product, prepare the PCR product for sequencing. Refer to the sequencing facility's guidelines for sample preparation and shipment.

DNA sequence analysis

Sequences from ITS, BT and TEF-1 α should only be compared to *D. helianthi* ex-type strain CBS 592.81, GenBank accession KC343115 (ITS), KC344083 (BT), and KC343841 (TEF-1 α). For a positive identification, the ITS sequence of the sample must be 100% match, and the BT and TEF-1 α sequences must be 99-100% match (no more than 3-bp differences) to the ex-type strain.

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