Investigating the host range and origins of *Phoma koolunga* (Ascochyta blight of field pea)

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Abstract

Ascochyta blight (synonym: blackspot) is a serious, globally distributed, primarily foliar disease of *Pisum sativum* L. (field pea). It is typically caused by a combination of three or four fungal species that can exist independently of each other, called a complex. *Phoma koolunga*, identified in 2009 in South Australia, is the most recent addition in the Ascochyta complex. Despite multiple international studies on Ascochyta blight of field pea, *P. koolunga* has not been reported anywhere else in the world and the origins of the pathogen, and if it occurs on other legume species remain unknown.

This study provides new information on the host range of *P. koolunga* on leguminous plants in controlled growth room conditions. To establish a host range, disease incidence and severity were assessed on 41 legume species comprising Australian native, weed, crop, pasture legumes and wild type *Pisum, Lathyrus* and *Vicia* species, following inoculation using two isolates of *P. koolunga*. All legumes tested, except *Cicer arietinum* (chickpea), developed leaf lesions and some also had stem and tendril lesions. Incidence and severity differed significantly among species but not consistently between isolates. The ability of the *P. koolunga* isolates to cause lesions on a wide range of legumes, including natives, in controlled environment conditions, suggests that it has a broad host range in humid and mild temperature conditions conducive for disease. Although all 17 native species developed some degree of leaf spotting, seven were considered susceptible because disease incidence was greater than 55 percent.

This research also details the isolation, identification and classification of *Didymellaceae* fungi causing leaf spots, collected from legumes during field studies undertaken to investigate a possible native origin of *P. koolunga*. Samples from plants with leaf spots were collected from field pea growing regions throughout New South Wales, South Australia and Victoria taken back to the laboratory and cultured. The resultant fungal isolates were identified based on both morphology and phylogenetic analyses of the internal transcribed spacer region and

part of the RNA polymerase II subunit B gene region. *P. koolunga* was not detected on native, weed or pasture legumes that had leaf spot symptoms in any of the regions visited, and only one isolate was recovered from field pea in the entire 2-year collection period. However, six novel species from the family Didymellaceae were isolated from Australian native legumes, five were from South Australia and one from New South Wales. The locations are represented by four different Australian Indigenous Peoples native language groups. Representatives of those groups were approached to request permission to use a suitable Aboriginal word for species epithet and permissions granted. These fungi are described here as *Didymella djirangnandiri* from *Swainsona galegifolia*, *Didymella kaurna* from *Gastrolobium celsianum*, *Neodidymelliopsis tinkyukuku* from *Hardenbergia violaceae*, *Nothophoma garlbiwalawarda* from *Senna artemisioides, Nothophoma naiawu*, and *Nothophoma ngayawang* also from *S. artemisioides*.

Additional findings from the field collections were the identification of three new host-pathogen associations for Australia. *Didymella pinodes*, the primary pathogen responsible for Ascochyta blight of field pea, was isolated from leaf spots on naturalised species *Vicia cracca* (tufted vetch) in New South Wales and on *Senna artemisioides* from five different locations across South Australia. The discovery that these legumes may serve as an inoculum reservoir hosts for *D. pinodes* has implications for epidemiology and management of Ascochyta blight of field pea because both commonly occur in field pea growing regions throughout South Australia. *Didymella lethalis* was isolated from naturalised species, *Lathyrus tingitanus* (tangier pea), growing in a creek bed located in a well-used recreation area in Adelaide, South Australia.

Phylogenetic analyses indicated that *P. koolunga* has a close relationship with the recently named species *Ascochyta boeremae* and supports the re-naming of *P. koolunga* as *Ascochyta*. Confirmation of the correction in nomenclature to *Ascochyta koolunga* **comb. nov.** was

achieved with PCR followed by sequencing at two additional loci, the partial gene regions of ß-tubulin and the partial large subunit nrDNA (LSU).

In summary, the controlled growth room results revealing a wide legume host range, and field collection results yielding no isolations from legumes other than field pea, suggest that *P. koolunga* is unlikely to have originated as a pathogen of Australian native legumes and provides no evidence regarding possible origins.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Abbreviations and Acronyms

ACT	Australian Capital Territory
AGG	Australian Grains Genebank
AIATSIS	Australian Institute of Aboriginal and Torres Strait Islander Studies
APDA	acidified Potato Dextrose Agar
APDD	Australian Pests and Diseases Database
BRIP	herbarium code for Queensland Plant Pathology Herbarium, Brisbane, Australia
CBS	herbarium code for CBS-KNAW, Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands
CER	controlled environment room
CLA	carnation leaf agar
DAR	herbarium code for NSW mycology and plant pathology herbarium, Orange, NSW, Australia
DI	disease incidence
DNA	deoxyribonucleic acid
dpi	days post-inoculation
DS	disease severity
FAO	The Food and Agriculture Organisation of the United Nations
GPS	global positioning system
GP3D	glyceraldehyde 3-phosphate dehydrogenase gene
GRDC	Grains Research & Development Corporation
GTR	general time reversible model
ITS	internal transcribed spacer region
LSU	partial large subunit nrDNA (28S, LSU)
MAFFT	**m**ultiple **a**lignment using **f**ast **F**ourier **t**ransform
MCMC	Markov chain Monte Carlo
MEA	malt extract agar
ML	maximum likelihood
NSW	New South Wales
NVT	national variety trial
OA	oat agar
ODAP	oxalyl diamino propionic acid
PCR	polymerase chain reaction

PDA	potato dextrose agar (full strength)
RAxML	Randomised Axcelerated Maximum Likelihood
RBG	Royal Botanic Gardens
RBGSyd	Royal Botanic Gardens - Sydney
rDNA	ribosomal deoxyribonucleic acid
RO	reverse osmosis
*rpb*2	RNA polymerase II subunit B
SA	South Australia
SARDI	South Australian Research and Development Institute
SBC	Sequence-based classification
SBI	Sequence-based identification
TreeBASE	repository of phylogenetic information produced and governed by The Phyloinformatics Research Foundation, Inc
TUB	300 bp of the ß-tubulin gene
*tub*2	partial gene regions of ß-tubulin
VIC	Victoria
VPRI	herbarium code for National Collection of Fungi, Bundoora Herbarium, Victoria, Australia
WA	Western Australia

Chapter 1

Introduction and Literature Review

1.1 Introduction

Field pea (*Pisum sativum* L.) is the second most important grain legume in the world after common bean. The Food and Agriculture Organisation of the United Nations (FAO) reported 8,141,031 hectares harvested, producing 16,205,448 tonnes of dry pea from 102 countries in 2017 (http://www.fao.org/faostat/, Kreplak et al. 2019). Australia was ranked 9th in the world with respect to area harvested and 10th for quantity produced. For yield however, Australia was placed 46th out of 102 countries. Contributing to yield loss in most years would be the disease Ascochyta blight (synonym: blackspot), a significant disease reported to occur in most field pea growing regions across the globe. Ascochyta blight reduces photosynthetic potential, which reduces seed set and weight, and causes accelerated plant maturity, seed staining and desiccation (Česnulevičienė et al. 2014). The result is significant reduction in pea quantity and quality (Tivoli et al. 2006, Tivoli and Banniza 2007).

Ascochyta blight is referred to as a disease complex because it is typically caused by a combination of three or four fungal species that can exist independently of each other: *Ascochyta pisi*; *Didymella pinodella* (syn. *Phoma medicaginis* var. *pinodella; Peyronellaea pinodella*); *Didymella pinodes* (syn. *Mycosphaerella pinodes, Peyronellaea pinodes*); and *Phoma koolunga*. The current preferred nomenclature for the second and third listed species is *Didymella pinodella* and *Didymella pinodes* (Chen et al. 2015a) so these names will be adopted in this thesis.

The principal pathogen within the Ascochyta blight complex reported in field pea growing areas in Australia, France, Canada, USA, New Zealand, China and India is *D. pinodes.* These countries also have *D. pinodella* (Davidson et al. 2009a, Le May et al. 2009, Liu et al. 2013, Liu et al. 2016, Panicker and Ramraj 2010). In Europe and North America, the third species in the complex is *A. pisi* (Ali et al. 1994, Chilvers et al. 2009, Le May et al. 2009, Onfroy et al. 1999). The third species in Australia, identified in 2009, is *P. koolunga* (Davidson et al. 2009a).

The contribution of *P. koolunga* to the infection levels measured in 2009 was 41%, with *D. pinodes* constituting 54%, and *D. pinodella* 5% (Davidson et al. 2009a).

Despite numerous international research projects on Ascochyta blight of field pea, *P. koolunga* has been detected only in Australia, which led to the overall aim for this research to investigate possible Australian origins of this fungus, perhaps in association with native legume species.

1.2 Field pea production and Ascochyta blight in Australia

Field pea was probably introduced into Australia in the late 19th century (Siddique et al. 2013). It was grown, including in South Australia (SA), on a limited scale until the 1980s. Production area increased until 2012 then remained steady (Siddique et al. 2013). In Australia Ascochyta blight is the most common and damaging disease in field pea (Salam et al. 2011c). Grain yield loss occurs regularly and has been reported to range from 15 to 75% (Bretag et al. 1995, Bretag et al. 2006, McMurray et al. 2011).

In 2016 the Grains Research & Development Corporation (GRDC) reported 232,100 hectares were sown to field pea, of which 125,000 hectares were in SA. In 2016 the Australian field pea crop had an export value of $80 million (Clarry 2016). The yield losses from Ascochyta blight cost the pulse industry millions of dollars annually. To reduce disease levels, the recommended guidelines include: a 5-year crop rotation period; sowing to avoid peak release of ascospores of the primary Ascochyta blight pathogen, *D. pinodes*, after rain; and planting non-infected seed (https://grdc.com.au/GN-Field-Pea-South 2017). While field cultivation can reduce soil-borne and pea stubble inoculum, most growers no longer cultivate, preferring direct seeding for stubble retention. Wet rainfall patterns and humid crop microclimates after sowing contribute to the Ascochyta blight disease burden. In wet conditions, potential yield losses are less readily controlled, making lower rainfall areas more

desirable to reduce disease (Lines et al. 2015). The frequency of isolation of all three Ascochyta blight pathogens from field pea seed has been reported as consistently low from crops grown in sandy loam soils where rainfall is also low (Bretag et al. 2006, Davidson 2012). Dressing seed with pre-emergent fungicide can be used but is not usually cost effective and foliar fungicides are only economic in field pea crops yielding over 2 tonnes per hectare (Salam et al. 2011a).

1.3 History of identification of *Phoma koolunga* in Australia

The first records of Ascochyta blight in Australia date from the 1960s (Ali et al. 1992). Lodgement of pathogen isolates and/or herbarium specimens in Australian fungal herbaria has occurred from the 1970s onwards. In Victoria (VIC) specimens are lodged in the Department of Primary Industry collection (VPRI) and in New South Wales (NSW) in the Department of Agriculture herbarium (DAR).

Two isolates in the NSW Department of Agriculture database, labelled DAR 67520 and 67521, were collected from *P. sativum* at Maitland, SA in 1978 and Freeling SA in 1976, respectively. In 1992 they were identified morphologically and submitted to NSW Department of Agriculture database as *Macrophomina phaseolina*. In 2009 a reclassification of both these isolates was made possible through the collection of Ascochyta blight samples over three years (2007-2009). Samples came from 13 pea fields in four geographical regions within SA; the Yorke Peninsula, Eyre Peninsula and Mid-North regions. PCR and sequencing of the rDNA internal transcribed spacer (ITS) region of these samples was used to produce a phylogenetic tree, which distinguished a new species in the Ascochyta complex, *P. koolunga*. DAR67520 and 67521 were not included in the ITS PCR and therefore not present in the phylogenetic tree, however, they were included in the morphological characterisation of *P. koolunga*, which supported their reclassification to *P. koolunga* (Davidson et al. 2009a). *P. koolunga* was

reported as belonging in *Phoma* section *Macrospora* using the definitions of Boerema et al. (2004), due to its large and occasionally 1-septate conidia (5–7 mm). There are only two other *Phoma* species from leguminous plants in this section, *Phoma boeremae* from *Medicago* spp. and *Phoma rabiei* which causes blight of chickpea. The conidia of both of those species are generally narrower than those of *P. koolunga*. In 2015, *P. boeremae* was reclassified as *Didymella boeremae* (Chen et al. 2015a).

The difficulty of classification of fungal species from field pea, based solely on morphology, before the advent of molecular techniques, has been demonstrated again more recently. Cultures isolated from *P. sativum* by G.H. Boerema at the Waite Agricultural Research Institute, SA, were deposited with MycoBank (Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands) in 1984. Known as isolates CBS 372.84 and CBS 373.84, they were originally deposited as *Ascochyta fabae*. These isolates have subsequently been shown to be distinct from the authentic cultures of *A. fabae* and in 2017 were described and renamed as a new species, *Ascochyta boeremae*, which is genetically closely related to *Ascochyta nigripycnidia* (Chen et al. 2017). Species of *Phoma* and *Ascochyta* have suffered and still do suffer from unclear taxonomic placement (Aveskamp et al. 2009, Aveskamp et al. 2010, Chen et al. 2015b, Chen et al. 2017, Kim et al. 2016). A close analysis of PCR sequence data at multiple loci for all the Ascochyta blight pathogens, including *A. boeremae,* to identify possible sequence synonymy is required.

Since 2009, research focusing on disease management and disease minimisation of Ascochyta blight in southern Australia has contributed information about the infection process and epidemiology of *P. koolunga*. (Davidson et al. 2011, Davidson et al. 2012, Davidson et al. 2013, Khani et al. 2016b, McMurray et al. 2011, Salam et al. 2011a, 2011b, Tran et al. 2014a and b, 2015a and b, 2017). In 2010, most field pea seed samples collected from pulse cropping areas in SA, VIC and Western Australia (WA) were infected by Ascochyta blight pathogens,

mostly *D. pinodes*. *Phoma koolunga* was isolated from samples from VIC and SA but not from WA. The frequency of isolation of *P. koolunga* from National Variety Trial (NVT) seed ranged from 0 to 6.3 % (Khani et al. 2016b). In the 2015 season *P. koolunga* was identified in more areas of the Eyre Peninsula region of SA, WA and VIC than in previous years (J.A. Davidson, 2016, *pers. comm.*). It is considered likely that the spread of *P. koolunga* has been due to seed transmission since seed harvested from naturally infected plants from several locations in SA and VIC was reported to be infected, and transmission to germinating seedlings in controlled conditions has been reported at 98%. The transport of seed stock by grain authorities and seed brokers for use interstate for research breeding purposes, or for the next season's crop, is common (Khani et al. 2016a). How much of the spread of infection can be attributed to infected seed as opposed to other sources like volunteer field pea plants from previous crops and potential alternative hosts needs investigation.

1.4 Ascochyta blight symptoms

The symptoms of Ascochyta blight caused by each fungal species in the complex are not easily distinguished and more than one of the species can be isolated from the same lesion. Symptoms begin as chlorotic spots which typically become necrotic and may expand and coalesce in favourable moist conditions. Symptoms can occur on leaves, stems and pods (Tivoli et al. 2006). Leaf spots can sometimes be attributed to particular fungi based on size and colour. Leaf spots caused by *A. pisi* alone are tan and circular. Leaf spots caused by *D. pinodes* and *D. pinodella* are multiple and purple brown in colour (Tivoli et al. 2006). Symptoms caused by *P. koolunga* on field pea leaves and stems were reported to be indistinguishable from those caused by *D. pinodes,* with the exception of delay in development by 24 hours (Davidson et al. 2009a).

Historically, *Ascochyta* species were considered to be leaf pathogens, whereas *Phoma* species were associated with stem lesions (Aveskamp et al. 2009). Symptoms of Ascochyta

blight can be observed at ground level but also below ground. Symptoms can include rot on the stem at ground level extending to invasive root lesions. Collar rot around the stem base has been reported to result from infected seed and is more commonly associated with *D. pinodella* than the other pathogens (Bretag et al. 2006, Tivoli and Banniza 2007). *D. pinodes, D. pinodella* and *P. koolunga* can all infect the roots and epicotyls of field pea seedlings causing necrotic lesions (Tran et al. 2015a). The type and location of Ascochyta blight symptoms assessed visually is not sufficient to distinguish the causal pathogen, especially as there could be more than one pathogen. To make the distinction between pathogens, phylogenetic analysis, supported by morphological characterisation, is the preferred combined method. However, using morphology combined with phylogenetic analysis revealed a complication to disease diagnosis, in that *Phoma herbarum, P. glomerata* and *Boeremia exigua* var. *exigua* were reported to be associated with lesions on field pea that looked like Ascochyta blight (Li et al. 2011, 2012, Tran et al. 2013, 2014a). For the four main Ascochyta blight pathogens, the location of symptoms on field pea, methods of dispersal and spread of disease are summarised in Table 1.1.

Table 1.1: Symptom location and dispersal methods of Ascochyta blight pathogens of field pea

Species name	Symptom location	Method of spread within and between crops	References
Ascochyta pisi	Leaf, stem and pods	Infected seed, field pea stubble, via conidia released with rain splash. Potential for ascospore dispersal is unknown	Ali et al. 1994, Bretag et al. 1995, Bretag et al. 2006, Chilvers et al. 2009, Davidson et al. 2009b, Onfroy et al. 1999, Skoglund et al. 2011, Tivoli and Banniza 2007
Didymella pinodes	Leaf, stem, pod spot, epicotyl rot in seedlings	Infected seed, field pea stubble, soil, volunteer plants, via conidia released with rain splash and released ascospores spread by wind	Ali et al. 1994, Bretag et al. 1995, Bretag et al. 2006, Chilvers et al. 2009, Davidson et al. 2009b, Onfroy et al. 1999, Skoglund et al. 2011, Tivoli and Banniza 2007
Didymella pinodella	Leaf, stem, pod spot, epicotyl rot in seedlings	Infected seed, field pea stubble, soil, via conidia released with rain splash	Ali et al. 1994, Bretag et al. 1995, Onfroy et al. 1999, Bretag et al. 2006, Tivoli and Banniza 2007, Chilvers et al. 2009, Davidson et al. 2009c
Phoma koolunga	Leaf, stem, pod spot, epicotyl and roots	Infected seed to seedling transfer, field pea stubble, soil, via conidia released with rain splash.	Ali and Dennis 1992, Davidson et al. 2009a, Davidson et al. 2011, Davidson 2012, Khani et al. 2016a, Khani et al. 2016b, Tran et al. 2015a

The symptoms of Ascochyta blight may be similar among the pathogens responsible but the severity of symptoms is influenced by a multitude of factors. Symptom severity can be affected by competition or a synergy between pathogens, but is also influenced by environmental conditions including climate and host genotype (Golani et al. 2016a, Le May et al. 2009, Le May et al. 2012, Onfroy et al. 1999, Wroth 1998). Additionally, microclimate, crop canopy architecture and plant age play a role in how successful pathogens can be in initiating infection then spreading within a crop (Richard et al. 2012, 2013). Crop management practices, like crop

rotation, destruction of infested field pea stubble and later planting to avoid release of conidia with rain, can decrease incidence and severity of disease (Khani, et al. 2016a, Salam et al. 2011c).

The differences observed in disease severity are also a measure of the natural distribution of aggressiveness within a population. A pathotype can be defined as a subclass, or group of isolates, distinguished from others of the same species by its virulence on a specific host (genotype) i.e., a qualitative difference in disease severity. In contrast, aggressiveness reflects the natural variation in virulence or level of disease (measured quantitatively) within the pathogen population and has been attributed to pathogenic diversity, whether true pathotypes or natural distributions in aggressiveness (Taylor and Ford 2007).

Specific interactions among isolates of the most prevalently cultured fungal species, *D. pinodes,* and field pea lines could not be identified by Wroth (1998) or by Onfroy et al. (1999). This agrees with the observations of Jha et al. (2012) that populations of this pathogen are characterized by a continuum of increasing aggressiveness rather than distinct virulence groups. It is generally accepted that resistance to Ascochyta blight is polygenic and there is no pathotype specificity (Tran et al. 2015b). The challenge to reducing the severity of infection is to develop field pea cultivars with levels of resistance to all of the *Ascochyta* pathogens through consideration of the dynamic pathogen populations over time and location, and not just to focus on *D. pinodes* (Tran et al. 2015b).

1.5 Ascochyta blight pathogens and alternative hosts

The subject of host range needs evaluation for its significance in inoculum production and the development of epidemics. The epidemiological importance of alternative hosts may be measured by the amount and timing of available, viable inoculum they supply. Ascochyta blight pathogens of legumes rely on four main sources of inoculum for spread; seeds, plant

debris, soil and volunteer plants (Table 1.1), (Tivoli and Banniza 2007). The role and extent to which volunteer plants are involved in providing an inoculum source of any of the Ascochyta blight pathogens from one field pea growing season to the next in Australia has not been explored in detail. A fifth source of inoculum not listed by Tivoli and Banniza is alternative hosts that can act as reservoirs, transferring inoculum to crop plants.

Plant pathogens exhibit different levels of host specificity (Frenkel et al. 2007). Pathogens only infecting specific crops are considered specialists whereas those fungal species infecting a wide range of plant species and producing symptoms are generalists (Le May et al. 2014, McDonald and Peck 2009, Milgroom and Tobin 2003, Trapero-Casas and Kaiser 2009). *Ascochyta* species causing Ascochyta blight on their respective legumes, including *A. pisi*, were considered host-specific specialists by Peever (2007) whereby plant species and fungal pathogens have had co-evolutionary interactions. An example of a close, almost exclusive, pathogenic relationship is *Phoma medicaginis* var. *medicaginis* isolated from *Medicago truncatula* (barrel medic), an annual medic widely grown in Australia. It exhibited a narrow host range, infecting only *M. truncatula* and *Medicago sativa* (alfalfa) and not the other legumes tested, which were field pea, lentils, faba bean, chickpea and lupin (Ellwood et al. 2006).

Despite the review paper by Peever (2007) regarding host specificity and Ascochyta blight of legumes, the host range data reported by other researchers for *A. pisi* suggest it can infect other legumes and is not specific to *P. sativum*. *Didymella pinodes* and *D. pinodella* do have a more extensive host range than *A. pisi* and the list of alternative hosts identified to date includes species of *Cicer, Lathyrus, Lens, Lupinis, Medicago, Melilotus, Phaseolus*, *Trifolium, Trigonella, Vicia* and *Vigna*. However, *A. pisi* has been reported to infect all the above species except for *Melilotus* and *Vigna* (Bretag et al. 2006, Miranda 2012).

The focus of the majority of host range studies of Ascochyta blight pathogens has been on the primary pathogen, *D. pinodes.* In a comprehensive investigation, Le May et al. (2014) reported a wide host range and significantly different disease severity among the legumes inoculated with *D. pinodes.* Of the species tested, field pea was the most susceptible, followed by clover, vetch, alfalfa and then faba bean. There were also significant differences among isolates within the same species, and cultivars responded differently to infection. Barilli et al. (2016) conducted an extensive host range examination and reported that *D. pinodes* can infect not only pasture legumes but also other legume crops potentially used in rotation with field peas. Such rotations could considerably increase the inoculum potential of the soil thereby affecting subsequent pea crops.

Medicago truncatula is a popular species to use in host range testing because it is an established model for legume genomic and genetic studies, with the entire genome sequence recently made available (Kang et al. 2016, Vaz Patto and Rubiales 2014). *Medicago truncatula* was included in host range studies for Ascochyta blight pathogens and Moussart et al. (2007) inoculated several accessions with *D. pinodes*, including nine from Australia. All accessions exhibited symptoms but leaf lesions were restricted, suggesting limitation of colonisation to initially infected cells caused by more effective phytoalexins in *M. truncatula* than produced by *P. sativum*. *Didymella pinodella* has also been documented to infect *M. truncatula* (Madrid et al. 2014, Rubiales et al. 2014).

Further investigations of the host range of the minor Ascochyta blight pathogen, *D. pinodella,* by Frenkel et al. (2007) found that it caused disease in *Cicer judiacium* and isolates were able to infect *Pisum fulvum* and *P. sativum*. Infection of fenugreek (*Trigonella foenum-graecum*) in VIC by *D. pinodella* was confirmed by morphological characterisation and sequence analysis of the rDNA ITS region. A comparison with GenBank sequences from the ITS region revealed that this region in two *D. pinodella* isolates from *P. sativum* differed by

only a single base from the isolate from fenugreek (Bretag 2005). Leaf spots on fenugreek caused by *Cercospora* and *Ascochyta* species and charcoal rot (*Macrophomina phaseolina*) have also been reported (Acharya et al. 2010). In 2016, BLAST searches of the NCBI nucleotide database with consensus sequences were performed on a fungal isolate from fenugreek in Canada. The sequences at the *G3PD* and *TUB* loci were a 100 % match for *D. pinodes* (Habibi et al. 2016). Fenugreek and field pea could therefore provide an inoculum source of *D. pinodella* and *D. pinodes* for cross-infection of each other if planted in close proximity, or in rotation. It needs to be determined if fungal isolates from each crop could cross-infect and if so how to minimise the risk of cross-infection. The susceptibility of these legumes to *P. koolunga* has not been investigated.

The more recently identified pathogens included in the Ascochyta blight complex in WA, namely *P. glomerata, B. exigua* var. exigua and *P. herbarum* (Lamichhane and Venturi 2015, Tran et al. 2013, 2014a) are all necrotrophic, generalist species and it was postulated that they can infect a range of host species using synergy. As such, synergy with other fungal species could be a strategy used to infect a wider variety of plants in different environments. Antagonism and/or synergy between pathogen species on the same host and the order of infection may influence the extent of disease. Host-pathogen relationships are always in flux and colonisation of non-host tissues might be a way for the fungi to survive while waiting to encounter the host on which they are pathogenic (Crous and Groenewald 2016).

There are relatively few reports of Ascochyta blight fungi attacking non-legumes and Bretag et al. (2006) considered any alternative hosts would be of minor importance where field peas are grown because of the inoculum readily available from soil and field pea stubble. However, current research recommendations for Ascochyta blight affected areas mean that field pea crops are grown in rotations which can include wheat, barley, oats, cereal rye, canola, chickpeas, faba beans, vetch or lentils. The aim is to minimise the accumulation of primary inoculum in soils

and on exposed field pea stubble, however in doing so increases the potential importance of alternative hosts as a source of inoculum.

While research has identified that both *D. pinodes* and *D. pinodella* have a broad host range, the host range data currently available for *P. koolunga* is limited. Ali and Dennis (1992) reported investigations of the host range of *Macrophomina phaseolina* (since identified as *P. koolunga*) in glasshouse conditions. Their isolates caused small leaf lesions on cultivars of *Medicago* sp. (pasture medics), *Lens culinaris* (lentil) and *Vigna mungo* (black gram or mungo bean). The legume host species tested were: *Cicer arietinum* cv. not specified (chickpea); *Lens culinaris* cv. Medik; *Lupinus albus* cv. Ultra (lupin); *Lupinus angustifolius* cv. Marri; *Lupinus luteus* cv. Yellow; *Medicago littoralis* cv. Harbinger (shore medic) and cv. Liosell; *Medicago sativa* cv. Hunter River (lucerne); *Medicago scutellata* cv. Miller (snail medic); *Trifolium subterraneum* cv. Woogenellup (sub clover); *Vicia faba* cv. Fiord (broad bean), and *Vigna mungo* cv. Hepper and *Vigna radiata* cv. not specified (mung bean). Of the species that showed symptoms, those on *V. mungo, M. littoralis* cv. Harbinger and *L. culinaris* were not typical of those produced on field peas and were only on leaves, not stems. Pycnidia were produced only on field pea stems. Davidson et al. (2009a) reported that *P. koolunga* produced small lesions on some genotypes of *L. culinaris*, *M. littoralis* and *M. scutellata* in the glasshouse. The entry for *P. koolunga* in the U.S. National Fungus Collections database lists *P. sativum* as the only known host (Farr and Rossman 2019). A comprehensive examination of the host range of *P. koolunga,* including Australian native legumes, remains to be conducted.

It is important to investigate more fully the host range of *P. koolunga* to identify any naturally infected alternative hosts that could provide potential sources of inoculum within and between field pea crops from one year to the next. *Phoma koolunga* was discovered in SA, therefore possible alternative host species might include Australian native legumes as well as naturalised introduced crop and pasture legumes occurring in the same geographical areas as

field pea in this state. Tivoli and Banniza (2007) reported that non-faba bean alternative hosts from borders and fields adjacent to faba bean crops were the source of Ascochyta blight within that crop and were much more important than infected seed. The role of alternative hosts in the disease cycle of Ascochyta blight of field pea caused by *P. koolunga* remains to be elucidated. A summary of legume hosts susceptible to the four main Ascochyta blight pathogens is presented in Table 1.2. This includes data taken from experiments conducted in controlled conditions conducive for infection, which may not occur in the field.

Table 1.2: Reported host range of Ascochyta blight pathogens

Host plant species	*Didymella pinodes*	*Ascochyta pisi*	*Didymella pinodella*	*Phoma koolunga*	References for one or more species *
Field pea	Yes	Yes	Yes	Yes	Ali and Dennis 1992, Barilli et al. 2016, Bretag et al. 2006, Davidson et al. 2009a, Hernandez-Bello et al. 2006, Le May et al. 2014, Tivoli et al. 2007, Trapero-Casas and Kaiser 2009
Lucerne/medics	Yes	Yes	Yes	Yes	Ali and Dennis 1992, Bretag et al. 2006, Davidson et al. 2009a, Hernandez-Bello et al. 2006, Le May et al. 2014, Moussart et al. 2006, Rubiales et al. 2014, Tivoli et al. 2007, Trapero-Casas and Kaiser 2009
Vetch	Yes		Yes		Ali and Dennis 1992, Bretag et al. 2006, Davidson et al. 2009a, Hernandez-Bello et al. 2006, Le May et al. 2014, Tivoli et al. 2007, Trapero-Casas and Kaiser 2009
Lathyrus	Yes	Yes	Yes		Gurung et al. 2002, Weimer 1947
Chickpea	Yes				Barilli et al. 2016, Bretag et al. 2006, Ellwood et al. 2006, Miranda 2012
Lentil	Yes	Yes	Yes	Yes	Ali and Dennis 1992, Bretag et al. 2006, Davidson et al. 2009a, Hernandez-Bello et al. 2006, Le May et al. 2014, Tivoli et al. 2007, Trapero-Casas and Kaiser 2009
Fenugreek	Yes		Yes		Bretag 2005, Habibi et al. 2016
Clovers	Yes	Yes	Yes		Ali and Dennis 1992, Bretag et al. 2006, Davidson et al. 2009a, Hernandez-Bello et al. 2006, Le May et al. 2014, Tivoli et al. 2007, Trapero-Casas and Kaiser 2009
Wild type pea	Yes	Yes	Yes		Abbo et al. 2007, Fondevilla et al. 2005, Golani et al. 2016b, Jha et al. 2012, Wroth 1998
Australian native legumes	Unknown	Unknown	Unknown	Unknown	NA

*Host range references for *P. koolunga* are Davidson et al. 2009b and Ali and Dennis 1992a only. However, Ali and Dennis in their paper identified their isolates as *M. phaseolina*, which were subsequently shown in 2009 to be *P. koolunga*.

1.6 Possible alternative hosts for *Phoma koolunga*

1.6.1 Native Australian legumes

No systematic inventory has been conducted of the biodiversity of fungi which may cause leaf spots on Australian native legume species. However, a review of the Australian Pests and Diseases Database (APDD) for *Phoma* species on Australian native plants revealed that several specimens had been recorded, as shown in Table 1.3. Unfortunately, all except for two isolates from *Platylobium* were herbarium specimens. The potential for the two historical *Phoma* species cultures obtained (VPRI13025a and VPRI13064a) to be *P. koolunga* was eliminated in the initial phylogenetic investigations and not investigated further. However, *Platylobium* species are potential candidates to investigate the possible indigenous origins of *P. koolunga* by including these legume species in host range experiments to ascertain susceptibility. A subsequent investigation could then target these species in field collections for culture and identification if leaf spots were observed.

Table 1.3: *Phoma* species recorded on Australian native legumes, adapted from Australian Pests and Diseases Database (APDD)*

Fabeacea/ Leguminosae Species	Catalogue Number	Scientific Name	Locality	State	Year - parsed
Hardenbergia	VPRI 16238b	*Phoma* sp.	Monbulk	VIC	not provided
Lathyrus	DAR-14046a	*Phoma* sp.	Five Dock	NSW	1965
Indigofera	VPRI 16168a	*Phoma* sp.	Mt. Waverley	VIC	not provided
Lotus	DAR-20579a	*Phoma* sp.	Coaldale	NSW	1970
Platylobium	VPRI 12960c	*Phoma* sp.	Mount Disappointment State Forest	VIC	not provided
	VPRI 13025a	*Phoma eupyrena*	Grampians National Park	VIC	not provided
	VPRI 13064a	*Phoma* sp.	Brisbane Ranges National Park	VIC	not provided
	VPRI 13407b	*Phoma* sp.	Cathedral Ranges State Park	VIC	not provided
Kennedia	VPRI 41624a	*Phoma* sp.	Grampians National Park	VIC	not provided
	DAR-43442b	*Phoma* sp.	Duffys Forest	NSW	1983

*https://www.planthealthaustralia.com.au/resources/australian-plant-pest-database

To consider growing native or other forage/pasture legumes within pea cropping areas necessitates research into their susceptibility to infection by *P. koolunga*, *D. pinodes* and *D. pinodella* and their ability to act as alternative hosts. One advantage of native Australian perennial legumes is that they are better adapted for low rainfall zones than existing cultivated species like field pea. Research into growth of native legumes as dual purpose crops (grain for human consumption, or animal grazing) has been conducted and the genera considered most suitable were *Cullen*, *Glycine*, *Glycyhrriza*, *Kennedia*, *Lotus*, *Rhynchosia*, *Swainsona* and *Trigonella* (Bell et al. 2012). The authors advised that adoption will only occur if the use of native species complements current profitable farming options and suggested use in areas of poor soil less suited to cropping.

1.6.2 Introduced pasture and forage legumes

It is important to determine if any of the field pea Ascochyta pathogens are pathogenic on alternative legume crops. Research on the suitability of *Lathyrus* and *Vicia* species as fodder legumes has increased since the early 1990s. *L. sativus* and *L. cicera* have a long history of use for human and animal consumption. They are suitable for low rainfall areas, have low production costs and provide superior yields of protein compared to field pea and faba bean. Both *Lathyrus* species have potential to be included as a legume in rotation with cereal crops.

Field trials with both *Lathyrus* species were conducted in 1995 on the Yorke Peninsula and at Roseworthy research facilities in SA, both field pea growing areas, to determine best sowing time, grazing suitability and grain recovery. Both *Lathyrus* species showed promise as a dual purpose forage/grain legume (Miyan 1995), although the grain of both *L. cicera* and *L. sativus* contains the neurotoxin, 3-(-N-oxalyl)-L-2,3-diamino propionic acid (ODAP), which can cause lathyrism, a paralysis of the lower limbs. The susceptibility of animal species to lathyrism is poorly understood. *L. cicera* is considered to have potential as a replacement ingredient in diets for growing pigs (Mullan et al. 1999). Both species also have potential to be used in a breeding program to improve the existing cultivars to provide a quality protein source and reduce levels of ODAP (Hanbury 2000). *Lathyrus sativus* cv. Ceora was reported to have disease tolerance superior to field pea and vetch; which disease was not specified but susceptibility to Sclerotinia white mould was noted. Ascochyta blight-like symptoms have not been reported (Handury et al. 2005).

Vicia sativa (common vetch) and *V. villosa* (purple vetch) varieties are multipurpose crops grown in rotation with cereals, mostly to control disease. Both species were reported to be infected, showing leaf spot symptoms, when inoculated with *D. pinodes* (Barilli et al. 2016). *Ascochyta viciae* can cause Ascochyta blight of these vetch species but most cultivars are

moderately resistant (Matic et al. 2015). It is not known if these *Vicia* species can be infected by *P. koolunga.*

Trifolium resupinatum (Persian clover) is a native of Turkey, Afghanistan, Portugal, Greece, Iran and Iraq, all countries that have field and wild type pea species. *Trifolium resupinatum* was introduced to SA in the 1950s and grown commercially in the early 1970s. It has become a valuable species for temperate pastures of southern Australia (https://www.dpi.nsw.gov.au/agriculture/pastures-and-rangelands/species-varieties/pf/factsheets/persian-clover). A South Australian cultivar called SARDI has been bred by the South Australian Research and Development Institute (SARDI) and is sold by the South Australian company, PastureGenetics, as a high-performance annual winter-growing fodder crop. No leaf spot pathogen is documented in the fact sheet pamphlet produced by PastureGenetics (2016) but it also does not state if anyone has observed leaf spots, or tested for susceptibility of cultivar SARDI, or any other cultivars, to Ascochyta blight pathogens.

The pasture medics *Medicago littoralis, M. polymorpha, M. sativa, M. scutellata* and *M. truncatula* have been included in legume host range studies for Ascochyta blight pathogens, primarily *D. pinodes,* as referenced in Table 1.2. Some of these species were also included in glasshouse experiments using *M. phaseolina* isolates (Ali and Dennis 1992). These are the isolates which have subsequently been shown to be *P. koolunga* (Davidson et al. 2009a). Results for *D. pinodes* showed the susceptibility of a number of *Medicago* species, albeit with symptoms not typical of Ascochyta blight. A systematic investigation of commonly occurring pasture medics is needed to determine if these species could act as alternative hosts for *P. koolunga*.

1.6.3 Wild type *Pisum* and *Lathyrus* species

Wild type relatives of crop species are commonly examined to assess the genetics of potentially advantageous phenotypic traits, including disease resistance. Murrell (2016) suggested that the

current global climate change results in shift and shrinkage of ranges for crop cultivation and the potential of wild relatives as an important source of genetic diversity for crop breeding is underestimated. Murrell (2016) also suggested that the practical use of wild peas is hampered by insufficient awareness of their diversity and how they differ from cultivated peas. Therefore, examination of useful traits including resistance to abiotic stress and pest and disease should be intensified.

Reviews of Ascochyta blight of field pea in 2002 and 2006 summarise historical research and conclude that the best sources of resistance are likely to come from primitive *Pisum* and *Lathyrus* species with resistance to stem and leaf infection under different control mechanisms (Bretag et al. 2006, Gurung et al. 2002). In field evaluations of cultivars of field pea for resistance to *D. pinodes,* growth habit, crop architecture and flowering time were strongly associated with disease severity, confirming that partial resistance is under polygenic control and is quantitative and moderately inheritable (Conner et al. 2012, Fondevilla et al. 2007, Fondevilla et al. 2008, Jha et al. 2012, Wroth 1998, Zhang et al. 2006). However, significant variation among and within species of *Lathyrus, P. sativum* and *P. fulvum* has been reported for leaf and stem infection (Gurung et al. 2002). In a study of molecular genetic variation in the genus *Lathyrus, L. ochrus* and *L. clymenum* were the most similar to *P. sativum* (Croft et al. 1999). Skiba et al. (2004) found two quantitative trait loci in *L. sativus* associated with *D. pinodes* resistance, suggesting that this species could be targeted for breeding with *P. sativum.*

The exact genetic origin of cultivated *P.* sativum species is unclear, which makes breeding decisions such as screening for sources of disease resistance more complicated. Ladizinsky (2015) described the genus *Pisum* as comprising two species; *Pisum fulvum* Sm and *Pisum sativum* L., with *P. sativum* being divided into three subspecies, ssp. *sativum* L., ssp. *elatius* Bieb. and ssp. *humile* Boiss. et Noe. *Pisum sativum* L. is the domesticated species and the two wild forms are the climbing peas ssp. *elatius* and ssp. *humile*, the latter of which comprises, in

turn, two races that originated in Israel, *P. sativum* ssp. *humile* var. *humile* and *P. sativum* ssp. *humile* var. *syriacum*. Added to these is another domesticated subspecies, *P. abyssinicum*, possibly derived from an *elatius* x *fulvum* hybrid and taken from the Near East to Ethiopia. It therefore occupies an intermediate position between *P. fulvum* and *P. sativum* and represents an independent domestication relative to *P. sativum.* However, the evolutionary phylogenetic picture is not yet unequivocal (Ladizinsky 2015).

Identification of resistance genes to the Ascochyta blight pathogens in wild type *Pisum* species was the aim of several investigations of *D. pinodes* (Conner et al. 2012, Fondevilla et al. 2005, Fondevilla et al. 2008, Gurung et al. 2002, Jha et al. 2012, Peever 2007, Wroth 1998). Breeding could then include genes found to confer some level of resistance in domesticated *P. sativum* varieties. Partial but increased resistance to infection by *D. pinodes* was found in *P. abyssinicum* and *P. fulvum*. Similarly, the derived hybrid subspecies of *P. sativum*, namely ssp. *sativum* var. *arvense*, ssp. *syriacum,* ssp. *elatius,* ssp. *transcaucasicum* and ssp. *asiaticum* also showed partial resistance to infection when compared to *P. sativum* (Fondevilla et al. 2005, Jha et al. 2012).

Golani et al. (2016a) studied isolates of *D. pinodes* originating from wild and domesticated pea in Israel and found *D. pinodes* was ubiquitous in wild populations of *P. elatius* and *P. fulvum*. However, the percentage leaf area with necrotic spots was very low, but higher on lower leaves, and infection was rarely observed on stems or pods, suggestive of some level of resistance. This is consistent with epidemiological observations of wild and cultivated field pea species in Israel by Dinoor (1974) who reported that Ascochyta blight did not reach epidemic proportions in wild *Pisum* populations but did cause major disease in cultivated field pea. There was no difference in aggressiveness among isolates taken from areas in Israel of dissimilar ecology, suggesting one metapopulation of *D. pinodes* in Israel (Golani et al. 2016a). It is not

known whether the Ascochyta blight pathogens were introduced to Israel and then spread into the wild, or were originally minor pathogens in the wild (Dinoor 1974).

It is unknown if *P. koolunga* can cause disease symptoms in wild *Pisum* or *Lathyrus* species. Inclusion of wild type pea species in host range investigations for Ascochyta blight pathogens, including *P. koolunga*, is necessary to assess if resistance to infection could indicate the presence of genes that could be of use in breeding less susceptible field pea cultivars.

1.7 Taxonomy and phylogenetics of Ascochyta blight pathogens

Identification of fungal species has been a problem when relying on morphology and host association only. All the fungal pathogens causing Ascochyta blight disease on cool climate legume crops belong to the Didymellaceae, an important family of Pleosporales, Dothideomycetes. The family, established by de Gruyter et al. (2009), has three main genera, *Ascochyta*, *Didymella* and *Phoma*. Recent revisions of phylogenetic relationships within the Didymellaceae have established more robust multi-locus phylogenies. Despite several investigations, their polyphyly remains unknown, meaning that any shared or similar characteristics could be the result of either multiple ancestral sources, or no common ancestors. *Phoma* is a highly polyphyletic genus with unclear species boundaries. The conventional systems of identification, namely morphological studies, chemotaxonomy, secondary metabolite and protein profiling, are functional but have limitations (Rai et al. 2014).

Molecular techniques are now routinely used to understand the genetic diversity and population structure of cultivated plants and important fungal pathogens, including *Ascochyta*, *Phoma* and *Didymella* (Aveskamp et al. 2010, Chen et al. 2015a, de Gruyter 2012, Hibbett et al. 2016, Salam et al. 2011a, Woudenberg et al. 2012). The classification of *Phoma* species and allied genera continues to be ambiguous and controversial. This is primarily due to morphological divergence and the lack of sequences being made available, thus making claims

to taxonomic placement of some genera in the Didymellaceae unable to be substantiated (Chen et al. 2017). However, increasingly DNA-based phylogenetic analyses and using a polyphasic approach to classify species have helped to delineate species relationships (Aveskamp et al. 2009, Aveskamp et al. 2010, Chen et al. 2015a, de Gruyter et al. 2009, de Gruyter 2012, Jayasiri et al. 2017).

Using a polyphasic approach involves both molecular and morphological characterisation of species. Sequence-based classification (SBC), which is the process by which species are discovered, named and classified according to their phylogenetic relationships, is combined with sequence-based identification (SBI), which is the process by which the products of taxonomy are used to identify individuals and determine the composition of communities with reference to existing classifications (Hibbett et al. 2016). Woudenberg et al. (2012) cautions against total reliance on SBI and promotes the value of complementary morphological investigation. Davidson et al. (2009a) used a polyphasic approach in a preliminary investigation to identify and compare a limited number of isolates of *P. koolunga* using the ITS locus sequence data. Current phylogenetics require inference, which requires expert decisions about data inclusion and analytical settings, and Hibbett et al. (2016) recommends improvements in the quality and consistency of data in the available databases to reduce limitations on usefulness. Since the preliminary study of *P. koolunga* in 2009 by Davidson et al. (2009a), revisions to *Phoma* taxonomy have occurred and additional information included in databases (Chen et al. 2015a, Chen et al. 2017). These developments necessitate a more detailed phylogenetic examination of an expanded number of *P. koolunga* isolates alongside neighbouring clades of Didymellaceae.

Molecular data have provided a better resolution of the phylogenetic and evolutionary development of *Phoma* species than morphology alone. The ITS locus is a commonly used standard for initiating phylogenetic identifications of fungi but additional molecular markers

such as DNA sequences encoding tubulin, actin and translation elongation factor have also been widely used by taxonomists to demarcate species (Rai et al. 2014). More recently, the RNA polymerase II subunit (*rpb*2) locus has been found to be one of the most informative for a number of fungi and one of the most successful in resolving species and identifying synonymy within the Didymellaceae (Chen et al. 2015a, Chen et al. 2017, Matheny et al. 2007, Reeb et al. 2004). When compared to the combined four locus concatenated tree using ITS, LSU, *rpb*2 and *tub*2, Chen et al. (2015a) found the typology of the single *rpb*2 phylogeny to be highly similar. The *rpb*2 gene is therefore regarded as superior for resolution at both species and generic level to ITS, LSU or *tub*2. Ahmed et al. (2015) describes molecular techniques as effective tools for fungal identification and taxonomy and therefore for distinguishing the different Ascochyta blight pathogens occurring in field pea crops in Canada. However, in that study neither *P. koolunga* or *A. pisi* were isolated and negligible isolations of *D. pinodella* were reported.

1.8 Identification and diversity in field collections of fungi

Investigations into fungal diversity have traditionally used morphological identification from cultures, fruiting bodies and spores. Increasingly, DNA and RNA sequencing is being used for phylogenetic analysis, and directly from environmental samples, for example soil or tissue (Hibbett et al. 2016). Using sequencing supported by morphological characterisation in biogeographical surveys of natural ecosystems to identify the isolates cultured, has resulted in the identification of novel species of fungi (Chen et al. 2015a, Chen et al. 2017, Crous et al. 2013, Hyde et al. 2016, Jayasiri et al. 2017, Laurence et al. 2015, Soleimani et al. 2018, Tan et al. 2016, Tan et al. 2018, Taylor et al. 2011, Valenzuela-Lopez et al. 2018), including species of Didymellaceae belonging to the genera; *Ascochyta, Didymella, Neodidymelliopsis*, *Nothophoma* and *Phoma.*

Identification of novel isolates expands the understanding of genetic variation among fungal species and can thereby contribute to decisions about potential sources for disease resistance. High levels of genetic variation within pathogens would suggest a better ability to overcome single or dominant gene resistance in plants and close genetic relationships between fungal species would indicate recent evolutionary divergence (Liu et al. 2013). A mating system that allows for sexual crossing is a mechanism that can generate high levels of genetic variation within fungal species. Development of a teleomorph is typically dependent on nutritional and environmental conditions (Tivoli and Banniza, 2007). Sexual fruiting bodies, pseudothecia, have been found in natural conditions for only one member of the Ascochyta blight complex, namely *D. pinodes*. Teleomorphs for *D. pinodella* and *A. pisi* have been reported on infected plant material in controlled conditions only (Chilvers et al. 2009, Onfroy et al. 1999, Tivoli and Banniza, 2007). Despite considerable effort to initiate formation of pseudothecia of *P. koolunga,* all attempts to date have been negative (Khani, 2014). Both genetic variation and relationships with the host are important factors in investigating host range and possible origins of species like *P. koolunga*.

1.9 Summary and aims of research

Ascochyta blight of field pea (synonym: blackspot) is a commonly occurring disease in Australia field pea crops, which usually consists of three fungal species in a complex capable of causing significant yield loss. In 2009, *P. koolunga* was found to contribute to the disease in SA. It is, however, now thought that *P. koolunga* has been present and causing Ascochyta blight in SA since the late 1970s but was incorrectly named at the time. Despite research since 2009 on many aspects of the disease in many field pea growing countries, *P. koolunga* has still only been isolated from Australian field pea crops, which has led to speculation that it might have origins in alternative hosts among Australian native legumes. This maybe because

researchers outside of Australia have not had cause to investigate specifically for the presence of *P. koolunga*, given the predominance of the other Ascochyta blight pathogens, which are readily isolated (JA Davidson, 2019, *pers. comm.*).

The host ranges for three of the pathogens in the disease complex, *A. pisi*, *D. pinodes* and *D. pinodella,* are relatively broad and include pasture and crop legumes, as well as species of *Lathyrus*, *Vicia* and wild type *Pisum*. To date, a comprehensive host range has not been established for *P. koolunga,* and native legumes with leaf spot symptoms have not been sampled to look for *P. koolunga*.

All the Ascochyta blight pathogens belong to the family Didymellaceae and reviews of the taxonomy of the pathogens have led to several taxonomic revisions and reclassifications. The evolutionary relationship among the Ascochyta blight pathogens is not clear, although they do share the timeline for appearance with their cultivated hosts. This is suggestive of a co-evolutionary history, but this needs further exploration, especially for *P. koolunga*.

The aim of the research presented in this thesis was to investigate the possible Australian origins of *P. koolunga*, perhaps in association with native legume species.

1.10 Linking statement

This research is presented in five chapters, including two manuscripts, Chapters 2 and 3, which have been prepared for publication in peer reviewed journals. As it is a requirement of the proposed journals to place table and figures for publication at the end of the manuscript this has been done for both these chapters. Numbering of tables and figures starts at one for Chapters 2 and 3. For Chapter 1, tables are numbered starting at 1.1 followed by successive number in increasing order and appear in context throughout the chapter. For Chapter 4 tables appear in context throughout the chapter, figures are numbered 1 to 3 and are at the end of the chapter. Appendix 3 contains additional tables and figures for Chapter 3, but which are not destined for publication in the manuscript, and numbering uses Roman numerals (Tables I to III, Figures I to VI).

Chapter 1 provides an introduction and a review of the literature on aspects of Ascochyta blight of field pea including; history of isolation and revisions to identification and nomenclature in Australia of the fungus *P. koolunga*, host range data and phylogenetic relationships among the Ascochyta blight pathogens.

Research on the host range of *P. koolunga* is presented in Chapter 2. Results are compared to the host ranges of the other Ascochyta blight pathogens and what this might suggest about the origins of *P. koolunga* in Australia is discussed. This manuscript will be submitted to Australasian Plant Pathology.

Chapter 3 reports field collections and the identification and classification of six novel species in the Didymellaceae from Australian native legumes using phylogenetic analysis and morphological characterisation. Phylogenetic evidence suggestive of a synonymy between *P. koolunga* and *A. boeremae* is presented, which links back to the history of isolation of *P. koolunga* in SA described in the literature review, Chapter 1. New host records for two known pathogens are presented. The implications for management of these fungi is discussed and

consideration given to the potential significance of naturalised legumes as alternative hosts. This manuscript will be submitted to MycoKeys.

Chapter 4 presents results for pathogenicity testing of the primary Ascochyta blight pathogen, *D. pinodes,* and one novel *Nothophoma* fungal species isolated from the Australian native *Senna artemisioides*.

Chapter 5 is a general discussion. The evidence gathered to explore the origins of *P. koolunga* is re-examined and the opportunities to further investigate the unanswered and new questions raised regarding the origins and classification of *P. koolunga* are discussed.

Chapter 2

Host range investigation of *Phoma koolunga*, a causal agent of Ascochyta blight of field pea

Host range investigation of *Phoma koolunga,* a causal agent of Ascochyta blight of field pea

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Abstract

Phoma koolunga is one of three species that cause Ascochyta blight (synonym: blackspot) of field pea (*Pisum sativum* L.) in Australia. *P. koolunga* was first described in 2009 from South Australian isolates and has since been reported in Western Australia and Victoria. However, the origin of the pathogen and its host range remain largely unknown. The aim of this study was to examine the host range of *P. koolunga* and to explore possible origin(s) of *P. koolunga* as a pathogen of field pea in Australia.

Host range experiments were conducted in controlled growth room conditions using a selection of 41 legumes comprising Australian native, naturalised, crop and pasture legumes, and wild type *Pisum, Lathyrus* and *Vicia* spp. Two isolates of *P. koolunga* were compared. Disease incidence and severity were measured as number of leaves and percentage of leaf area diseased every 4 days up to 12 days post-inoculation. All legumes tested, except *Cicer arietinum* (chickpea), developed leaf lesions and some also had stem and tendril lesions. Incidence and severity differed significantly among species but not consistently between isolates. The ability of the *P. koolunga* isolates to cause lesions on a wide range of legumes in controlled environment conditions suggests the fungus has a broad host range in humid and mild temperature conditions conducive for disease. These results also suggest that *P. koolunga* has not evolved with one particular Australian native legume and is, therefore, unlikely to have origins in Australian native legumes. These results provide no evidence regarding the origins of *P. koolunga.*

Keywords *Pisum sativum*, disease incidence, disease severity, Australian native legumes

Introduction

Field pea (*Pisum sativum* L.) is thought to have been introduced into Australia in the late 19th century and was grown with other winter pulse crops on a limited scale until the 1980s, when planting increased as improved varieties became available (Siddique et al. 2013). Ascochyta blight (synonym: blackspot) was first recorded in Australian field pea crops in the 1960s (Ali and Dennis 1992). Ascochyta blight is a serious, globally distributed, primarily foliar disease of field pea. In Australia, it is associated with grain yield loss of 15 to 75% (Bretag et al. 1995, Bretag et al. 2006, McMurray et al. 2011).

Ascochyta blight may be caused by a combination of up to four fungal species that can exist independently of each other in what is referred to as a disease complex. The species are; *Ascochyta pisi*, *Didymella pinodella* (syn. *Phoma medicaginis* var. *pinodella, Phoma pinodella, Peyronellaea pinodella*), *Didymella pinodes* (syn. *Mycosphaerella pinodes, Peyronellaea pinodes*) and *Phoma koolunga*. The principal pathogen of the Ascochyta blight complex reported in field pea growing areas in Australia, Canada, China, France, India, New Zealand and USA is *D. pinodes,* and *D. pinodella* is typically present also (Davidson et al. 2011, Le May et al. 2009, Liu et al. 2013, Liu et al. 2016, Panicker and Ramraj 2010). In Europe and North America, the third species in the complex is *A. pisi* (Chilvers et al. 2009, Le May et al. 2009, Onfroy et al. 1999); although this fungus has been isolated in Australia it is not common. The common third species in Australia is *P. koolunga* (Davidson et al. 2009).

P. koolunga was first identified in South Australia (SA) and characterised by Davidson et al. (2009a). To date, it has been isolated only from Ascochyta blight-affected field pea in Australia. However, Ali et al. (1982) previously reported infection of field pea seed samples by *Macrophomina phaseolina*, which is now thought to have been *P. koolunga* (Davidson et al. 2009a). Research on *P. koolunga* since 2009 has focused on aspects of the epidemiology, infection process, disease management and disease minimisation, and has contributed to an

understanding of the Ascochyta disease complex in southern Australia (Davidson et al. 2009a, Davidson et al. 2011, Davidson et al. 2012, Khani et al. 2016a and b, Tran et al. 2014a and b, 2015a and b, 2017). The origin of *P. koolunga* is unknown.

Investigating the host range of *P. koolunga* and comparing it with the host range of the other Ascochyta blight pathogens has the potential to improve understanding of the role of alternative hosts in disease development. Barilli et al. (2016) concluded from an extensive host range study that *D. pinodes* can infect not only pasture legumes but also other legume crops potentially used in rotation with field peas. Infested stubble of alternative hosts could result in disease in subsequent field pea crops. Additionally, knowledge of the susceptibility of legume species to *P. koolunga* could facilitate the identification and incorporation of genetic material from resistant species into *P. sativum*. Identification of susceptible native legume species could help to explain the origin of *P. koolunga* in SA, as *P. koolunga* might have evolved as a pathogen or endophyte of a native legume which was subsequently able to infect field pea.

To date, limited host range investigations were reported by Ali and Dennis (1992), who tested isolates of *M. phaseolina* (now presumed to be *P. koolunga,* Davidson et al. (2009) from *P. sativum* in glasshouse conditions. Species and cultivars tested included: *Cicer arietinum; Lens culinaris* cv. Medik; *Lupinus albus* cv. Ultra, *L. angustifolius* cv. Marri, *L. luteus* cv. Yellow; *Medicago littoralis* cv. Harbinger and cv. Liosell, *M. sativa* cv. Hunter River, *M. scutellata* cv. Miller; *Trifolium subterraneum* cv. Woogenellup; *Vicia faba* cv. Fiord; *Vigna mungo* cv. Hepper, and *V. radiata*. Small lesions developed on leaves of cultivars of *L. culinaris* (lentil), *Medicago* spp. (pasture medics), and *V. mungo* (black gram or mungo bean). Symptoms on *L. culinaris, M. littoralis* cv. Harbinger and *V. mungo* were not typical of those produced on field peas and occurred on leaves but not stems. A comprehensive examination of the host range of *P. koolunga,* including Australian native legumes, remained to be conducted.

In Australia, native perennial legumes have the potential to be developed for pasture and grain since they are often better adapted for low rainfall zones than existing cultivated species like field pea. The genera considered most suitable as dual purpose crops are; *Cullen, Glycine, Glycyrrhiza, Kennedia, Lotus, Rhynchosia, Swainsona* and *Trigonella* (Bell et al. 2012). Investigating the susceptibility of these genera to *P. koolunga* is worthwhile to assess any risk they may pose for field pea if developed as pasture or grain crops. Likewise, naturalised weed and pasture legume species as well as Australian native legumes occurring in the same geographical areas as field pea cropping may be potential hosts for *P. koolunga*. The aim of this research was therefore to conduct a systematic investigation into the host range of *P. koolunga* as a pathogen on crop, pasture, naturalised and Australian native legumes occurring naturally in field pea growing regions of SA to identify; (i) susceptible species that might help to explain the origin of *P. koolunga* in South Australia, (ii) species which might serve as alternative hosts of *P. koolunga,* and (iii) evaluate some accessions which might be useful in resistance breeding programs.

Materials and Methods

Fungal isolates

Two single conidium-derived isolates of *P. koolunga* from the South Australian Research and Development Institute (SARDI) culture collection were used; FT07026 (collected 2007, field pea trial site, Pinery, SA) and isolate 2, FT01511 (collected 2015, field pea trial site, Riverton, SA), designated isolates 1 and 2, respectively. These isolates had been deemed moderately aggressive to field pea in the greenhouse (Davidson et al. 2009a, Khani et al. 2016b). Both isolates were passaged through *P. sativum* cv. Kaspa before use (see Appendix 1). Mycelial plugs of each isolate retrieved from storage at 4 ºC were placed onto potato dextrose agar (PDA, Oxoid™) plates and incubated under 12 h black and fluorescent light/12 h darkness cycle at 22

ºC. After 12-14 days, conidial suspensions were prepared and adjusted to 1.5×10^5 conidia/mL. Tween 20 (0.01%) was added as a surfactant (Davidson et al. 2012) .

Legume species

Plant species were selected based on one or more of the following criteria: native legume species on which a *Phoma* sp. fungal specimen has been registered in the Australian Pests and Diseases database and of which viable cultures were available; natural geographic distribution within field pea cropping regions in SA; adequate viable seed available; ability to germinate and grow in a controlled environment room (CER); legume species or genus of interest for development for forage or human consumption; known to be susceptible to *D. pinodes*.

Preliminary experiments were conducted to assess the viability of a wide range of pasture and native legume seeds to identify the conditions and times required to germinate and produce plants sufficiently large for inoculation in the CER. There was insufficient seed to assess viability of wild type species. Based on prior testing of time to germination of the wild type species, seed was sown 17–20 days prior to inoculation after scarification. Preliminary experiments were also conducted to determine day and night temperatures suitable for plant growth by adjusting the lighting and the temperature of the CER.

A total of 41 legume species were chosen after the preliminary experiments (Table 1). Genera of crop and pasture legumes were; *Cicer, Lathyrus, Lens, Medicago, Pisum, Trifolium* and *Vicia.* Australian native genera were; *Cullen, Glycine, Glycyrrhiza, Goodia, Hardenbergia, Indigofera*, *Lotus, Platylobium, Senna, Swainsona, Templetonia, Tephrosia* and *Trigonella.* The native legume species *Senna artemisioides* ssp. *quadrifolia*, *Daviesia genistifolia, D. leptophylla* and *Kennedia prostrata* were excluded due to poor germination (0 to 10%).

Seed of *Medicago* and *Trifolium* spp. was obtained from the SARDI Australian Pastures Genebank, Adelaide, South Australia. A small selection of wild *Pisum*, *Lathyrus* and *Vicia* species was chosen from the Australian Grains Genebank (AGG) in Horsham, Victoria, based on availability of sufficient seed and to provide a globally diverse spread with respect to seed origin. Australian native legume seed was obtained from the Australian Pastures Genebank, the Kersbrook Landcare Group (Kersbrook, SA) and Blackwood Seeds (Victor Harbor, SA). All seed was stored at 4º C prior to planting.

Seed preparation, planting and growth conditions

Due to space limitations within the two plastic humidity tents (160 L × 83 W × 83 D cm) in the CER, the 41 legume species were divided into two groups, planted and inoculated in two consecutive experiments, 21 species in experiment 1 and 20 in experiment 2. Both experiments included *P. sativum* as a positive control. Pots (6 × 6 × 4 cm) were filled with BioGro™ soil (van Schaik Pty Ltd, SA). Seed coats of Australian native species were scarified by rubbing gently between two sheets of #60 grade sandpaper. *P. sativum* was sown, four seeds per pot, 14 –16 days prior to inoculation to allow growth to the 3 to 4-leaf stage. *Trifolium*, *Medicago, Vicia* and *Lathyrus* spp. were sown between 17 and 21 days prior to inoculation. Some native species were grown for up to 11 weeks prior to inoculation due to variability in germination and growth habit.

Experiment 1 was conducted as a completely randomised block design, with seven pots of each species (three inoculated pots for each isolate and one non-inoculated control pot). The 120 pots for Experiment 1 were arranged onto eight trays, 15 pots per tray, four trays in each plastic humidity tent to be inoculated with an individual isolate. The pots with plants destined to be the non-inoculated controls were put onto separate trays in each tent, and carefully placed to provide no opportunity for leaves to touch those of inoculated plants.

Temperature was measured hourly throughout the experiment using a Tinytag™ data logger, both before and after inoculation. The CER was maintained at 15 ºC for the 12-h dark period and was set to 10 ºC for 12-h light period. The lights above the tents consisted of five 1000W globes running at 60% capacity, resulting in a temperature within the plastic tents of between 20 and 23 ºC during the day cycle and 15 ºC at night. Tents were closed up to time of inoculation except for watering when needed. After Experiment 1, the plastic tents were surface-disinfected with 70% ethanol and Experiment 2 was conducted using the same randomised block design and growth conditions as Experiment 1.

In both experiments, poor germination of some species led to variation in numbers of pots, plants and leaves available for inoculation, resulting in 7 of the 41 species having fewer than four plants per pot. Seedling emergence of *H. violacea, P. fulvum, P. obtusangulum, S. planticola, S. canescens, T. shaerospora* and *V. narbonensis* was sub-optimal. From seven pots per species planted with four seeds, one to ten plants were produced. Despite the total comprising 41 plant species, both experiments consisted of 120 pots because of the poor germination. Where there were only three pots, one pot was used for isolate 1, one for isolate 2 and one control. Only one pot sown with *P. fulvum* and two pots of *P. obtusangulum* produced seedlings. As *P. fulvum* has been reported to have some resistance to other Ascochyta blight pathogens, it was decided to include this one plant and inoculate with a single isolate without a control (Barilli et al. 2016). The poor germination left space in the randomised blocks of 60 pots for additional pots. To fill the gaps in the randomised blocks in Experiment 1, three pots of *V. sativa* were included and inoculated with isolate 2 only. To fill the gaps in Experiment 2, an additional three pots of *S. purpurea* were included in each of the two tents, inoculated separately with the two isolates and *V. sativa* was include again and inoculated separately with the two isolates. Repeating the inoculation of *S. purpurea* and *V. sativa*, in addition to *P. sativum*, served as a further check of repeatability.

Plant inoculation

At the time of inoculation, trays were removed from the tents and CER onto one trolley for each isolate and moved to separate areas for inoculation to avoid cross-contamination of isolates. Trays of control pots were kept on a separate trolley. Suspensions of conidia were applied using a hand-held garden sprayer, on three pots of each plant species per isolate unless otherwise stated. In addition, non-inoculated control plants (one pot) of each species were sprayed with sterile reverse osmosis water supplemented with 0.01% Tween 20. All plants were sprayed until run-off, approximately 3 mL per pot.

After inoculation pots were returned to tents within the CER in the conditions already described. For the first 48 h post-inoculation a humidifier (ionmax™ Andatech Pty Ltd) was used in each tent at medium level II mist setting to provide humid conditions conducive to infection and disease development.

In Experiment 1 the legume species inoculated with isolate 1 were placed in tent 1 and those inoculated with isolate 2 were placed in tent 2, and *vice versa* for Experiment 2 to check for consistency.

Disease assessment

Plant response to inoculation was visually assessed at days 4, 8 and 12 days post-inoculation (dpi). Disease was assessed as incidence (DI) and severity (DS). Twenty randomly chosen inoculated leaves per pot were assessed, 10 if fewer than 20 leaves present at inoculation. To reduce sampling bias the same method of choosing leaves was used at each timepoint by assessing leaves in clockwise order around the pot, working from outside to inside to obtain the 20 or 10 inoculated leaves per pot (Fondevilla et al. 2005). The estimated DS was entered directly into the smart-phone application, PMapp, a tool for recording estimates of percentage area affected (Birchmore et al. 2015). PMapp includes standard area diagrams, designed to

assist data collection, and a self-calibration function to test accuracy. Prior to visually assessing disease, a self-calibration test was conducted that generated an accuracy score of 96% for the assessor. From the 10 or 20 assessments, PMapp calculated % DI and % DS per pot. Photographs were taken of all species at 4-day intervals post-inoculation.

To satisfy Koch's postulates, leaf samples from inoculated plants showing leaf spot symptoms were collected at 12 dpi. Leaves were surface disinfected following the method of the Royal Botanic Gardens Sydney (M. Laurence, 2017, *pers. comm.*). In a laminar flow cabinet, leaves were sprayed with 70% alcohol and blotted dry with tissue paper from a newly opened box. Leaf segments of approximately 5 × 3 mm were placed on acidified PDA (APDA) containing 1 mL of 85% lactic acid/L of PDA to minimise bacterial contamination and incubated as described above for 10 days, when resulting fungal colonies were identified. Macroscopic colony morphology characteristics and size of conidia were compared to published descriptions of *P. koolunga* (Davidson et al. (2009a).

Statistical analysis

Disease responses

DI and DS assessments of each plant species and each isolate were regressed against days post-inoculation using the standard regression package within R statistical software (RCoreTeam 2014). After 8 days, senescence of some species reduced the number of leaves available for assessment. After 8 dpi, growth of new, non-inoculated leaves on some species confounded disease assessment. Therefore, the fitted values of the regressions at day 8 were considered to be the most discriminatory for assessing disease among legume species and between isolates. Use of the fitted value from the regression gave a consistent measure that was unaffected by the number of observations (scores of DS on the 10 or 20 inoculated leaves per pot) recorded on each day.

The data consisted of DI and DS for the 20 or 10 leaves assessed in each replicate pot at each time point. For each set of 20 or 10 leaves (per pot) a straight line was fitted to the data (regressed against dpi) and the discriminating value at day 8 was taken as a summary of DI or DS per pot. The average and standard error (SE), at day 8, for each pot were calculated and data were plotted on a scale of 0 to 1 where 0 = no disease and 1=100%. DI and DS for the three additional pots of *S. purpurea* and *V. sativa* included in Experiment 2 were subjected to the same regression analysis and SE calculated to examine any significant difference in DI and DS between experiments for these two species. Zero disease data from mock-inoculated water controls were not included in the analyses.

DI and DS at day 8 were plotted against each other for each species using a broken-stick or piecewise regression model (Toms and Lesperance 2003). This is used where two or more lines are joined at unknown points, called breakpoints, that show change in slope or transition point. For this research the breakpoint marks the transition from resistant to increasing degrees of susceptibility to infection by *P. koolunga*.

Results

Differences in disease incidence and severity between isolates

Plant species tested showed a similar response to the two isolates in terms of the average DI and DS at 8 dpi ($P > 0.05$) for isolates 1 and 2 calculated for plant species individually in each experiment (Figs 1 and 2). However, in two species, *G. lotifolia* and *L. odoratus,* isolate 1 resulted in a significantly greater DI than isolate 2 ($P < 0.05$). Isolate 1 caused more severe disease on *P. obtusangulum* whereas isolate 2 caused more severe disease on *L. cicera* and *M. sativa* than did isolate 1 ($P < 0.05$). These results that are inconsistent with the majority could be explained by variability in inoculation technique or disease assessment, poor plant health, minor difference in plant age or low numbers of inoculated leaves to score. For the purposes

of subsequent analysis, it was assumed that there was likely to be no overall significant difference between isolates. The DS and DI results at 8 dpi were reproducible for the species included in both experiments (*P. sativum, S. purpurea, V. sativa*); there was no significant difference in DI or DS ($P > 0.05$) between experiments. The response of *P. sativum* was similar in both experiments. The DI on *P. sativum* in both experiments was 90-100% and DS was 38-64%, depending on isolate and experiment.

Susceptibility of legume species to Phoma koolunga

DI and DS scores on the 41 legume species inoculated with *P. koolunga* differed significantly among species ($P < 0.005$) (Figs 1 and 2). The scale on the x and y axis for Figs 1 – 4 is proportional, 0.0 to 1.0, which equates to 0% to 100% DI or DS. To enable comparisons with similar literature for the other Ascochyta blight pathogens we will report results as the equivalent percentage. For the purpose of comparison of plant species, and because neither isolate consistently caused greater DI and DS than the other, data for the two isolates have been combined for the following analysis.

Cicer arietinum was the only species tested that did not show necrotic lesions. DI and DS at 8 dpi were highest on *Pisum* spp. (range of means DI = 85-99%; DS = 24-61%), of which *P. abyssinicum* showed the most severe disease and *P. fulvum* the least. DI and DS were also high on *Lathyrus cicera* but much less so on *L. odoratus* (mean DI = 89% and 45%; DS = 34% and 7%, respectively). *T. resupinatum* showed moderate incidence and severity (mean DI = 48%; DS = 30%). *Lens culinaris* had a high incidence but low severity (mean DI = 74%; DS = 14%). *Medicago* spp. showed wide variation (range of mean DI = 27-92%; DS = 3-27%), with disease most severe on *M. littoralis* and *M. sativa*. *Vicia* spp. were also variable (range of mean DI =12-49%; DS = 0.03-7%). In contrast to the other *Vicia* species tested, *V. sativa* had many internode lesions.

All of the Australian native species tested exhibited disease, but symptoms differed widely. In decreasing order according to average DS (data for two isolates combined) at 8 dpi; *S. purpurea* was most susceptible (DS = 47%) followed by *T. retusa* (DS = 33%), *T. sphaerospora* (DS = 31%), *S. canescens* (DS = 29%), *P. obtusangulum* (DS = 25%), *T. suavissima* (DS = 22%), *G. lotifolia* (DS = 17%), *I. australis* (DS = 13%), *L. australis* (DS = 10%), *G. acanthocarpa* (DS = 7%), *S. pleurocarpa* (DS = 5%), *G. clandestina* (DS = 3%), *S. plantiicola* (DS = 3%), *G. tabacina* (DS = 2%), *G. canescens* (DS = 2%), *H. violacea* (DS = 0.3%) and *C. australasicum* (DS = 0.1%).

The average DI and DS at 8 dpi for the two isolates on each species were combined per experiment and plotted against each other. The resulting scatter plots (Figs 3 and 4) show a transition breakpoint at 0.55 (55%) for Experiment 1 and 0.69 (69%) for Experiment 2. The difference in breakpoint was attributed to the combination of legume species tested in each experiment. The results for *P. sativum* in each experiment were consistent. Of the 41 legumes species tested, 22 were considered susceptible, falling after the breakpoint. The susceptible species were; all eight *Pisum* spp., eight of the 17 native legumes (*S. purpurea, S. canescens, T. retusa, P. obtusangulum, I. australis, L. australis, G. lotifolia, T. suavissima*), three *Lathyrus* spp. (*L. sativus, L. cicera, L. ochrus*) and three pasture legumes (*T. resupinatum, M. littoralis*, *M. sativa*). The most resistant species, with respect to both incidence and severity determined by the breakpoint, were *C. arietinum* followed by *C. australasicum* (mean DI = 12%, DS = 0.1%), *H. violacea* (mean DI = 11.5%, DS = 0.3%), *V. narbonensis* (mean DI = 12%, DS = 0.3%) and *V. benghalensis* (mean DI = 28.0%, DS = 0.2%).

Qualitative differences in disease symptoms were observed but no one particular type of lesion was exclusive to one host species. Examples of five types of leaf lesion are shown in Fig. 5a-f. Dry, circular lesions, with dark brown rims and light brown centres, that led to senescence and leaf drop, were observed on *G. lotifolia* (Fig. 5a). Similar but more uniformly

brown necrotic lesions that continued to spread but did not cause leaf drop were observed on *L. ochrus* (Fig. 5b). Light brown blotches, sometimes following leaf veins, were exemplified by *S. purpurea* (Fig. 5c). Small, localised tan or chlorotic spots were observed on *L. odoratus* (Fig. 5d). Small necrotic leaf lesions were observed on *V. benghalensis* and *C. australasicum* (Fig. 5e and f, respectively).

The incidence and severity of lesions on other plant parts was not quantified, however, symptom development on nodes, internodes, tendrils or stems was recorded photographically. Lesions on other plant parts developed more slowly than on leaves and were most conspicuous and severe on *Pisum* spp. (Fig. 6a) followed by *T. sauvissima, S. purpurea, I. australis* (Fig. 6b) and *V. sativa* (Fig. 6c).

Koch's postulates were fulfilled. *P. koolunga* was isolated from all surface disinfected leaf samples from each species and its identity confirmed.

Discussion

The findings presented in this research demonstrate, for the first time, that *P. koolunga* can cause necrotic leaf spots on a range of Australian native legumes. That *P. koolunga* also infected species of *Medicago, Trifolium, Vicia, Lathyrus*, wild *Pisum* and *Lens* confirms and extends the previous report by Ali and Dennis (1992) of symptoms on *L. culinaris* and *M. littoralis.* However, differences in plant age need to be borne in mind when considering relative susceptibility

C. arietinum (cv. Howzat) was the only species to remain asymptomatic following inoculation. Likewise, chickpea was not infected in the experiments reported by Ali and Dennis (1992). However, it is possible that other cultivars of *C. arietinum* inoculated with different isolates of *P. koolunga* could prove susceptible. Barilli et al. (2016) reported responses of *C.*

arietinum to *D. pinodes* to vary greatly, depending both on isolate and accession tested, and one of the isolates tested failed to infect any of the accessions.

The two isolates of *P. koolunga* tested were broadly similar in pathogenicity. In comparison, Khani et al. (2016b) reported differences among three *P. koolunga* isolates following inoculation of four *P. sativum* genotypes. Likewise, Le May et al. (2014) reported significant differences in disease severity within individual plant species following inoculation with four isolates of *D. pinodes*. In the present study, there were only five instances where DI or DS differed significantly between isolates 1 and 2. This low level of difference could be attributed to variability in spray inoculation, in visual disease assessment or, in the case of *P. obtusangulum*, the small number of plants available for inoculation, rather than actual differences in isolate aggressiveness and disease. It is likely that testing a larger range of isolates of *P. koolunga* could reveal differences in aggressiveness.

Disease incidence and severity differed significantly among the 41 legume species. Analysis using the piecewise regression model showed a breakpoint when disease incidence reached between 55 to 69 % after which there was a steep increase in incidence and severity, indicating a transition from relatively resistant to susceptible species. A total of 22 species were considered susceptible and 19 were more resistant. Using piecewise regression as a tool for identifying thresholds could prove useful in genetic testing for Ascochyta blight resistance in field pea.

Le May et al. (2014) reported differences in disease severity among the crop and pasture legumes inoculated with *D. pinodes.* Of the species tested, field pea was the most susceptible, followed by *Trifolium* sp., *Vicia sativa*, *Medicago* sp. and then *Vicia faba*. In the present research, a decreasing order of susceptibility to *P. koolunga* would comprise *Pisum* as most susceptible and *Vicia* species as least, and intermediate would be *Trifolium, Lathyrus* and

Medicago spp. The results of this research mirror those of Le May et al. (2014) in that *M. sativa* and *V. sativa* were the most susceptible of the species tested within that genus.

D. pinodes can cause lesion on barrel medic (*M. truncatula*), common vetch (*V. sativa*) and fenugreek (*Trigonella foenum-graecum*) (Barilli et al. 2016, Habibi et al. 2016, Madrid et al. 2014, Moussart et al. 2007, Rubiales et al. 2014) and *D. pinodella* causes lesions on barrel medic (Rubiales et al. 2014). The current study showed that *P. koolunga* is also able to cause leaf and stem lesions on barrel medic, common vetch and sweet fenugreek (*Trigonella sauvissima)* and was reisolated from these lesions. These legume species could therefore be reservoirs of inoculum for the Ascochyta blight pathogens found in Australia.

Several studies of wild type *Lathyrus* and *Pisum* species have identified potential resistance to Ascochyta blight. Weimer (1947) reported *L. tingitanus* to be moderately resistant and *L. sativus* resistant to *Ascochyta pinodella* (*D. pinodella*) and *Mycosphaerella pinodes* (*D. pinodes*). However, all attempts to cross these plants with the winter field pea varieties available at that time failed. The results of the present study suggest that the choice of *Lathyrus* species for use in *Pisum* breeding will be important because the five *Lathyrus* species tested varied significantly in disease severity following inoculation with *P. koolunga.* While only one accession of each species was used here, *L. odoratus* had the greatest resistance to infection by *P. koolunga*, followed by *L. clymenum* and *L. ochrus. L. sativus* and *L. cicera* were most susceptible (DI = 72% and 89%; DS = 14% and 34%, respectively). Gurung et al. (2002) reported significant variation in leaf and stem infection by *D. pinodes* among and within species of *Lathyrus*, *L. ochrus* being less susceptible than *L. sativus* and *L. clymenum. L. sativus* is grown as a fodder crop in Australia and, as a potential alternative host for *P. koolunga,* cropping in rotation with, or near, field pea should be reviewed.

In this research, all wild *Pisum* species tested were susceptible to *P. koolunga.* Least susceptible was *P. fulvum* (DI = 85%; DS = 24%) followed by *P. sativum*, *P. arvense, P.*

elatius, P. transcaucasicum, P. pumilio and, most susceptible, *P. abyssinicum* (mean DI = 99%; DS = 62%). Barilli et al. (2016) concluded that cultivated *Pisum* species were more susceptible than *P. fulvum* to *D. pinodes* and reported DS from 7 to 67%. Although only one plant of *P. fulvum* was inoculated with *P. koolunga,* the DS was 24% whereas *P. sativum* had DS of 40%, indicating similarity to the results of Barilli et al. (2016) for *D. pinodes*. In comparison, other researchers using *D. pinodes* have reported partial but increased resistance to infection in; *P. fulvum, P. sativum* (subspp. *asiaticum, elatius, sativum* var. *arvense*, *syriacum, transcaucasicum*) and *P. abyssinicum* when compared with *P. sativum* (Conner et al. 2012, Fondevilla et al. 2007, 2008, Jha et al. 2012, Wroth 1998, Zhang et al. 2006). Our observation that *P. koolunga* caused disease on *P. elatius* would, however, support the conclusion of Weimer (1947) that *P. elatius* was not a suitable source of genes for resistance to *Ascochyta pinodella* (*D. pinodella*) and *Mycosphaerella pinodes* (*D. pinodes*).

In a field survey of wild type *Pisum* accessions, Golani et al. (2016a) reported *D. pinodes* to be ubiquitous in wild populations of *P. elatius* and *P. fulvum* in Israel, however, disease typically comprised sparse necrotic spots. When accessions of wild field pea were screened against *D. pinodes* in Western Australia, the most useful germplasm, which displayed partial resistance, originated largely from Turkey, followed by Afghanistan, Ethiopia and Greece (Siddique et al. 2013). *Pisum* accessions tested in the current study originated from the former Soviet Union, Georgia, Afghanistan, Morocco, Turkey and England. The results presented in this study suggest that, in the limited number of wild type *Pisum* accessions tested, the *P. koolunga* isolates used caused more severe disease than has been reported for *D. pinodes* isolates in similar experiments. A direct comparison of a number of isolates of *D. pinodes* and *P. koolunga* would be needed to test this suggestion.

The inoculum concentration used in this study (1.5×10^5 conidia per mL) was consistent with or less than similar host range studies conducted in controlled environments using *D.*

pinodes and *D. pinodella* (Barilli et al. 2016, Le May et al. 2014). However, it is acknowledged that the amount of inoculum used was likely to be greater than would be encountered in Ascochyta blight-affected field pea crops. Consequently, while this research answers some questions regarding the legume species which may serve as hosts for *P. koolunga* it does not mean that these species can be infected in the field and, consequently, exhibit disease.

This investigation also sought to elucidate the possible origins of *P. koolunga*, discovered in SA, within native, weed and pasture legumes. All 17 native Australian legumes tested showed leaf spot symptoms when inoculated with *P. koolunga.* However, the statistical analysis revealed a wide range of susceptibility. While *Pisum* spp. were among the more susceptible, it is not known why seven of the 17 Australian native legumes would be among the more susceptible. The most susceptible native legume was *S. purpurea* (DS = 42%) and the least susceptible were *C. australasicum* and *H. violacea* (mean DS = 0.1 and 0.3%, respectively). The native legumes tested in the study were chosen because they were indigenous to the field pea growing areas but the susceptibility results alone do not provide direct clues to the possible origins of *P. koolunga* in SA. The results do suggest that growing susceptible native legumes in proximity to areas with a history of *P. koolunga* might result in infection of the young native legumes if there were sufficient inoculum and humidity favourable for disease development.

Pasture legumes such as *V. sativa*, *Medicago* spp. and *T. resupinatum* can be found in and around field pea crops during the winter growing season, continue to grow in summer and can be infected by three Ascochyta blight pathogens. Both pasture and native legume species could act as potential inoculum sources for Ascochyta blight infection of field pea and could be of epidemiological importance in determining location and crop rotations with field pea.

The Ascochyta blight pathogens *D. pinodes* and *D. pinodella* have extensive host ranges that include species of *Cicer, Lathyrus, Lens, Lupinis, Medicago, Melilotus, Phaseolus*,

Trifolium, *Vicia* and *Vigna* (Barilli et al. 2016, Bretag et al. 2006, Le May et al. 2014, Miranda 2012). The results of this study suggest that *P. koolunga* also has a broad host range that includes Australian native legumes. It is not known if *D. pinodes* or *D. pinodella* could cause leaf spot disease on Australian native legumes. The wide host range of *P. koolunga* would suggest that it has not evolved with, nor originated from, Australia native legumes. *P. koolunga* is more likely to be an agriculturally introduced pathogen of as yet unknown origins. A field survey is being undertaken to investigate if *P. koolunga* can be identified in association with disease on pasture and Australian native legumes in field pea growing regions of southern Australia.

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Tables and Figures

Table 1: Legume species tested in host range experiment

Species Number	Botanical name	Common name	Origin	SARDI Australian Pastures Genebank or AGG ID number
1	*Cicer arietinum*	Chickpea cv. Howzat	SARDI 2015 field trial, South Australia	NA[1.]
2	*Cullen australasicum*	Native scurf-pea	Kersbrook, South Australia	NA
3	*Glycine tabacina*	Vanilla glycine	Rutherglen Research Institute Vineyard, Victoria, Australia	41680
4	*G. clandestina*	Twining glycine	Red Hill region, South Australia	41515
5	*G. canescens*	Silky glycine	mid north, South Australia	41345
6	*Glycyrrhiza acanthocarpa*	Native liquorice	Renmark, South Australia	9536
7	*Goodia lotifolia*	Golden tip	Victor Harbor, South Australia	NA
8	*Hardenbergia violacea*	Purple coral pea	Nuriootpa, South Australia	41625
9	*Indigofera australis*	Australian Indigo	no origin provided	40367
10	*Lathyrus cicera*	Red pea	Australia	80521
11	*L. clymenum*	Crimson pea	no origin provided	80987
12	*L. ochrus*	Cyprus vetch	Greece	80110
13	*L. odoratus*	Sweet pea	Yates commercial seed, Australia	NA
14	*L. sativus*	Chickling pea	Bangladesh	80719
15	*Lens culinaris*	Lentil cv. Flash	SARDI, 2015 field trial, South Australia	NA
16	*Lotus australis*	Austral trefoil	Elliston, South Australia	35129
17	*Medicago littoralis*	Strand medic cv. Harbinger	SARDI, South Australia	NA
18	*M. polymorpha*	Burr medic cv. Scimitar	SARDI, South Australia	NA
19	*M. sativa*	Lucerne	SARDI, South Australia	NA
20	*M. scutellata*	Snail medic cv. Kelson	SARDI, South Australia	NA
21	*M. truncatula*	Barrel medic cv. Paraggio	SARDI, South Australia	NA

22	*Pisum fulvum*	wild pea ancestor	no origin provided	3732
23	*Pisum sativum*	Field pea cv. Kaspa	SARDI, 2015 field trial, Minlaton, South Australia	NA
24	*P. sativum* subsp. *abyssinicum*	not recorded	Morocco	3308
25	*P. sativum* var. *arvense*	not recorded	England	2315
26	*P. sativum* subsp. *asiaticum*	not recorded	Afghanistan	5387
27	*P. sativum* subsp. *elatius*	not recorded	Georgia	1663
28	*P. sativum* var. *pumilio*	not recorded	Turkey	2309
29	*P. sativum* subsp. *transcaucasium*	not recorded	Former Soviet Union	2380
30	*Platylobium obtusangulum*	none recorded	Kersbrook, South Australia	NA
31	*Senna plantiicola*	Yellow Pea	Port Augusta, South Australia	41109
32	*Senna pleurocarpa*	Smooth cassia	Maralinga, South Australia	41110
33	*Swainsona canescens*	none recorded	Kingoonya, South Australia	41038
34	*S. purpurea*	none recorded	Moon Plain, South Australia	342815
35	*Templetonia retusa*	Cockie's tongues	Calca, South Australia	41113
36	*Tephrosia sphaerospora*	none recorded	Roxby Downs, South Australia	42712
37	*Trifolium resupinatum*	Persian clover	SARDI, South Australia	NA
38	*Trigonella suavissima*	Sweet fenugreek	Marree, South Australia	42744
39	*Vicia benghalensis*	Purple vetch	Former Soviet Union	62827
40	*V. narbonensis*	Narbon bean	Lebanon	60122
41	*V. sativa*	Common vetch cv. Morava	SARDI, South Australia	NA

[1.] NA – Not Applicable

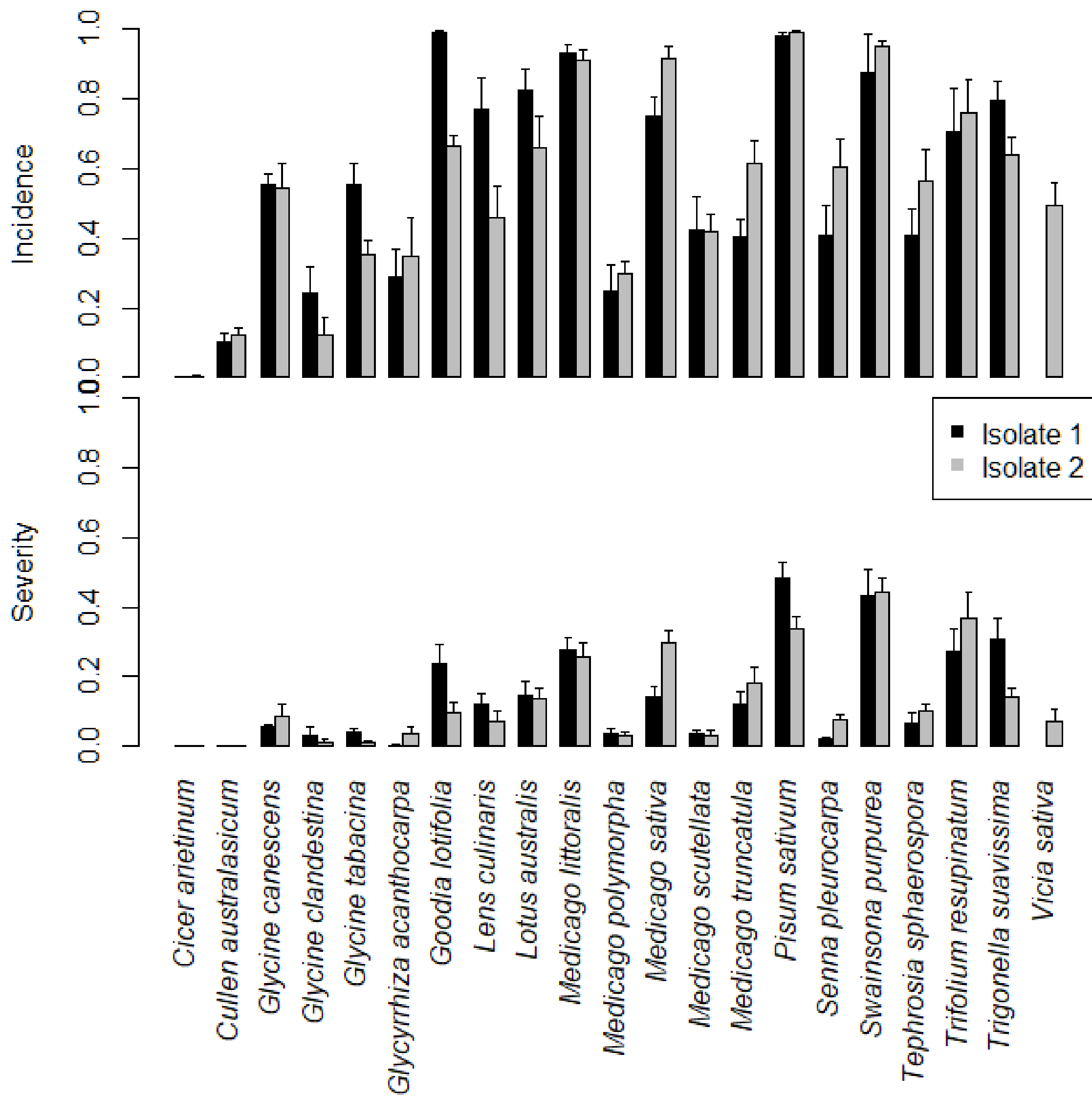

Fig. 1 Experiment 1, disease incidence (DI) and disease severity (DS) on 21 species of leguminous plants 8 days after inoculation with two isolates of *Phoma koolunga* in a controlled environment room. Isolate 1 FT07026, isolate 2 FT01511. 0.0 = 0%, 1.0 = 100%, error bars represent standard error (SE) from the sample mean, descriptive of the random sampling process.

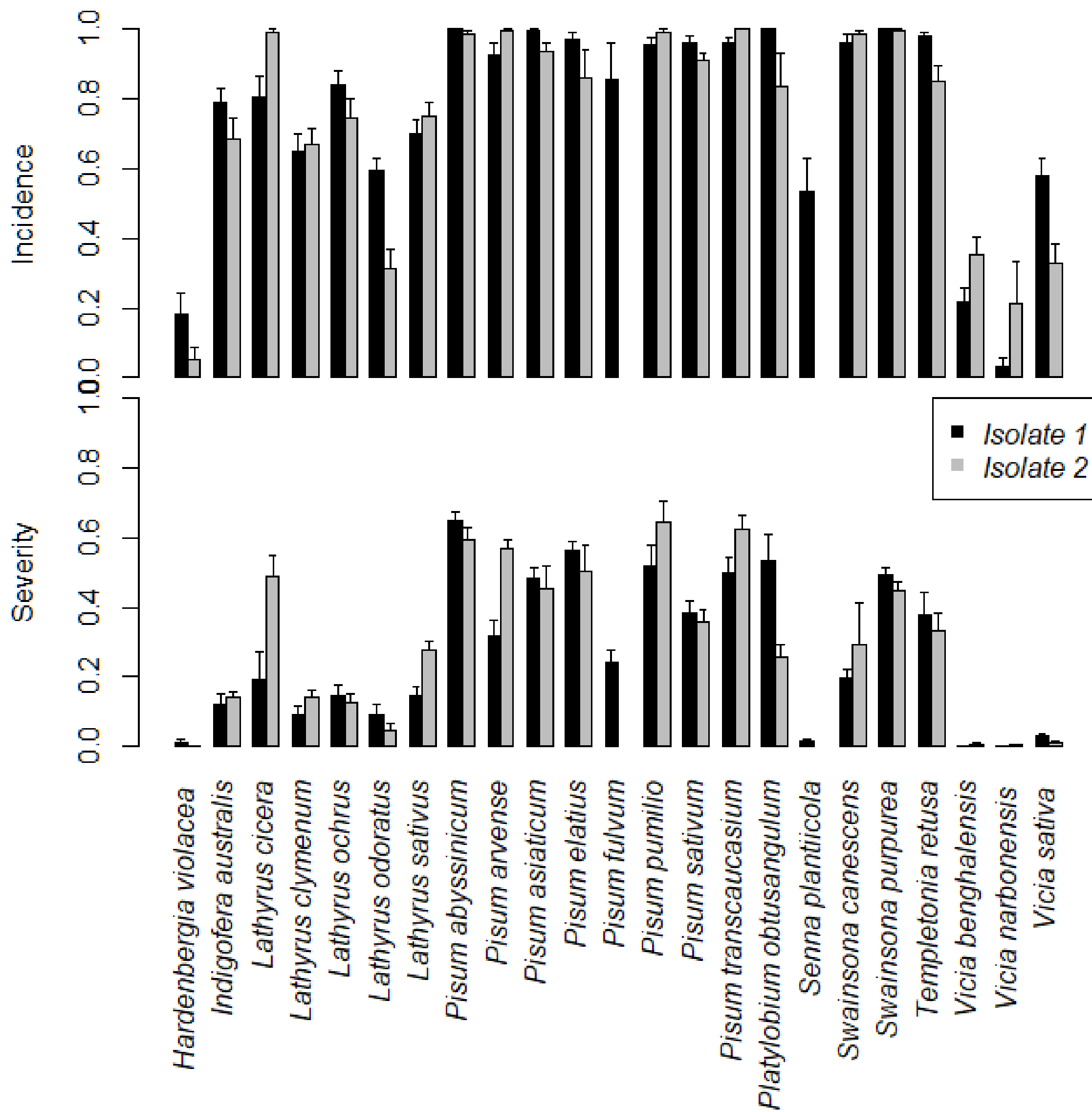

Fig. 2 Experiment 2, disease incidence (DI) and disease severity (DS) on 23 species of leguminous plants 8 days after inoculation with two isolates of *Phoma koolunga* in a controlled environment room. Isolate 1 FT07026, isolate 2 FT01511. 0.0 = 0%, 1.0 = 100%, error bars represent standard error (SE) from the sample mean, descriptive of the random sampling process.

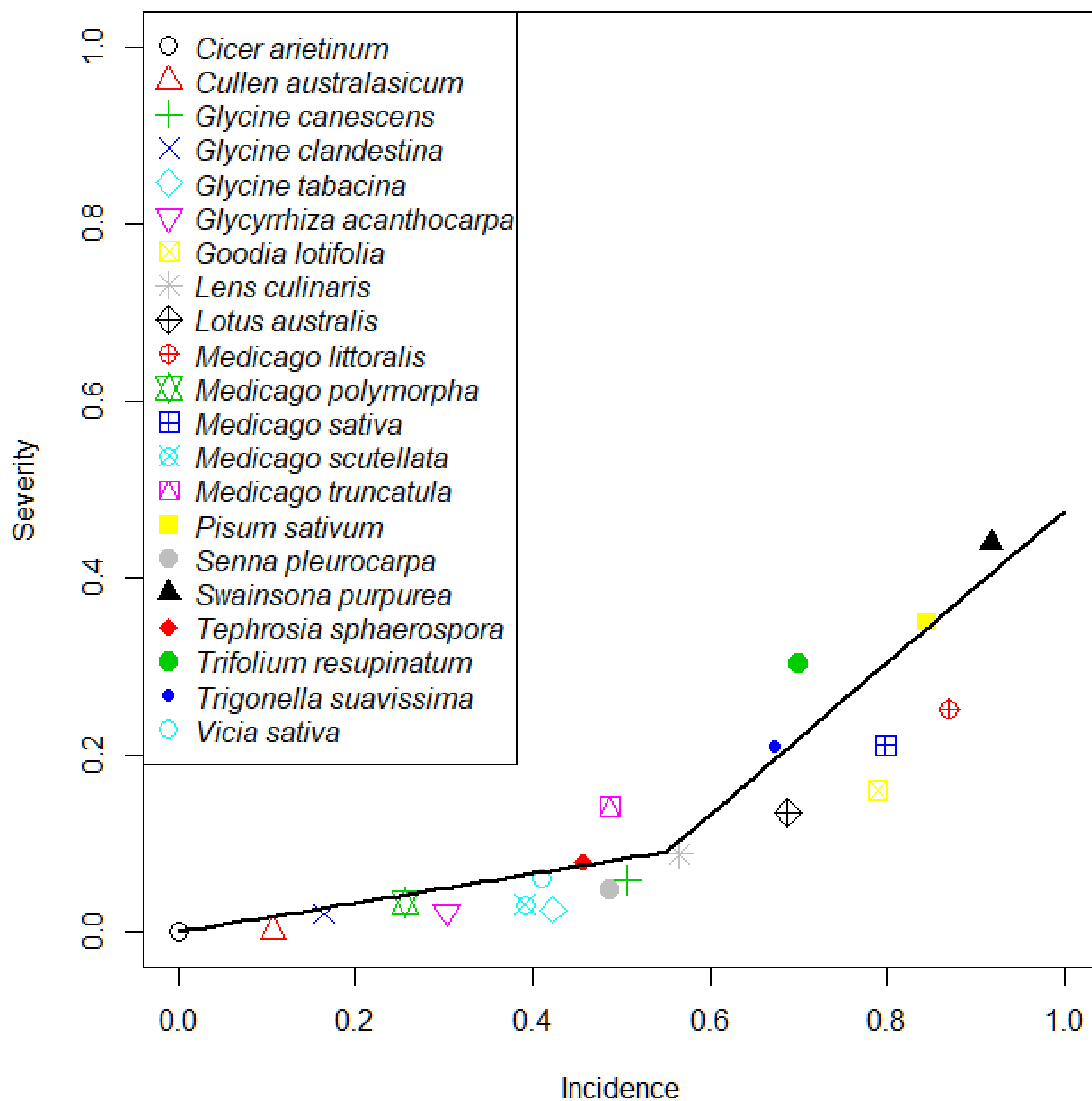

Fig. 3 Disease severity *versus* disease incidence of leguminous species inoculated with *Phoma koolunga* in Experiment 1. Data for disease incidence and severity at 8 days post-inoculation for both isolates combined. 0.0 = 0%, 1.0 = 100%. Breakpoint at 0.55 marks transition from more resistant to increasingly susceptible within this group of plant species tested.

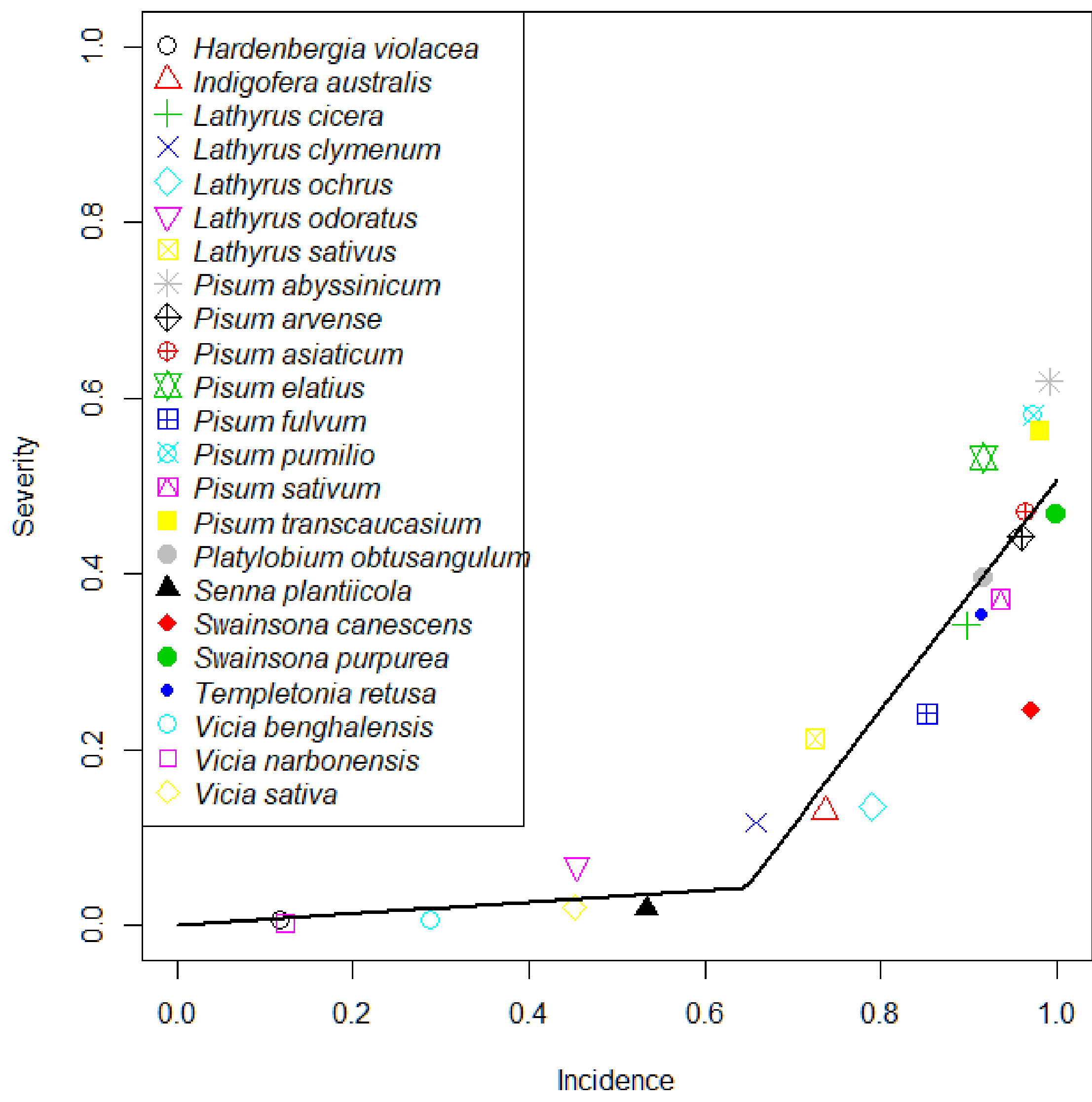

Fig. 4 Disease severity *versus* disease incidence of leguminous species inoculated with *Phoma koolunga* in Experiment 2. Data for disease incidence and severity at 8 days post-inoculation for both isolates combined. 0.0 = 0%, 1.0 = 100%. Breakpoint at 0.65 marks transition from more resistant to increasingly susceptible within this group of plant species tested.

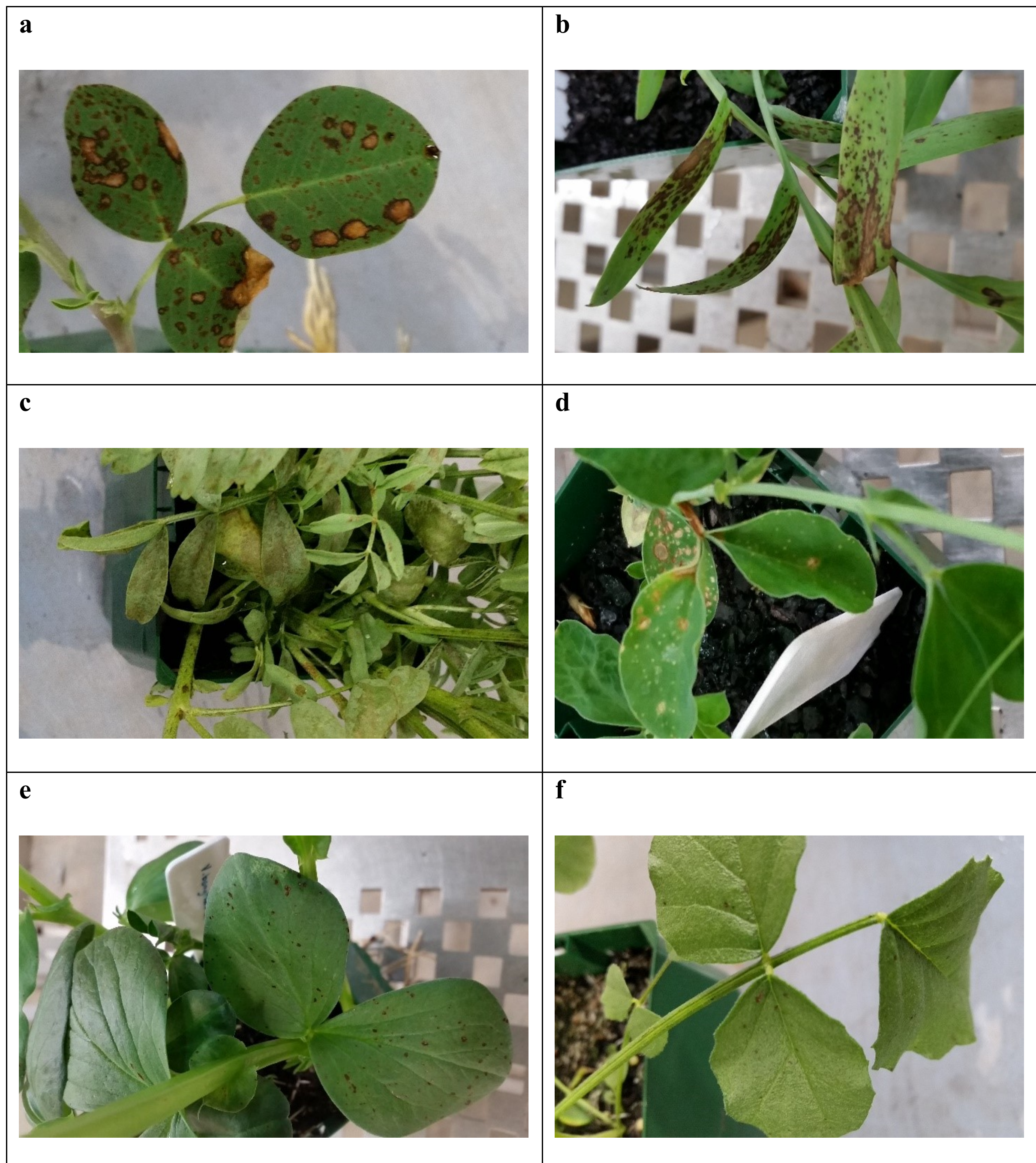

Fig. 5 Symptoms on a range of legumes 8 days after inoculation with *Phoma koolunga* in a controlled environment, **(a)** circular brown necrotic leaf lesions with dark margins on *Goodia lotifolia*, **(b)** blotchy necrotic leaf lesions on *Lathyrus ochrus,* **(c)** light brown leaf and stem lesions on *Swainsona purpurea,* **(d)** tan and chlorotic leaf lesions on *Lathyrus odoratus,* **(e)** small necrotic leaf lesions on *Vicia benghalensis* **(f)** small necrotic leaf lesions on *Cullen australasicum*.

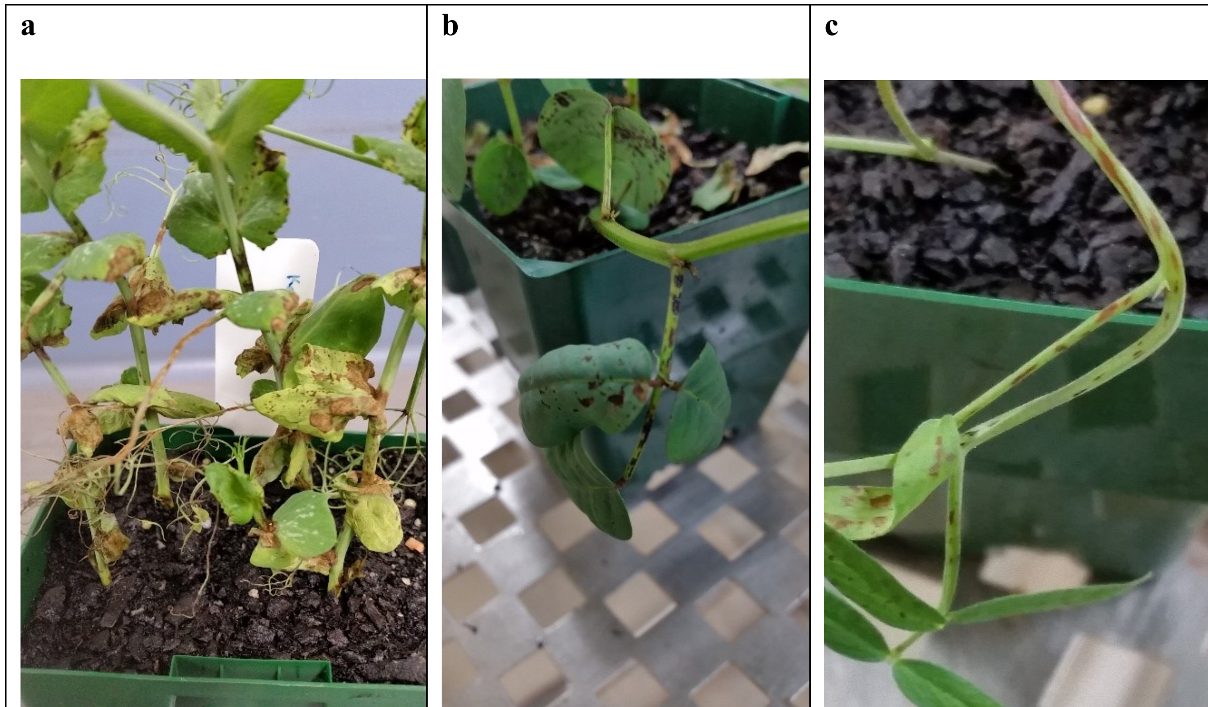

Fig. 6 Examples of species susceptible to leaf and stem infection 8 days after inoculation with *Phoma koolunga* in a controlled environment, **(a)** light brown necrotic stem, tendril, petiole and leaf lesions on *Pisum sativum* (cv. Kaspa), **(b)** dark brown leaf and stem lesions on *Indigofera australis,* **(c)** light reddish brown leaf and stem lesions on *Vicia sativa.*

Chapter 3

Cryptic diversity found in Didymellaceae from Australian native legumes

Cryptic diversity found in Didymellaceae from Australian native legumes

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Abstract

Ascochyta blight of *Pisum sativum* (field pea) in southern Australia is typically caused by a combination of up to four fungal pathogens that can exist independently of each other. One of these species, *Phoma koolunga* (Didymellaceae, Pleosporales), was first described and identified in 2009 as a causal agent of Ascochyta blight in South Australia. *Phoma koolunga* has not been reported anywhere else in the world, and its origins and occurrence on other legume (Fabaceae) species remain unknown. Blight and leaf spot diseases of Australian native, pasture and naturalised legumes were studied to investigate possible native origin of *P. koolunga*.

Six novel species in the Didymellaceae were isolated from leaf spots of Australian native legumes from commercial field pea regions throughout southern Australia. The novel species were classified on the basis of morphology and phylogenetic analyses of the internal transcribed spacer region and part of the RNA polymerase II subunit B gene region. Three of these species, *Nothophoma garlbiwalawarda* **sp. nov.**, *Nothophoma naiawu* **sp. nov.** and *Nothophoma ngayawang* **sp. nov.**, were isolated from *Senna artemisioides*. The other species described here are *Didymella djirangnandiri* **sp. nov.** from *Swainsona galegifolia*, *Didymella kaurna* **sp. nov.** from *Gastrolobium celsianum*, and *Neodidymelliopsis tinkyukuku* **sp. nov.** from *Hardenbergia violaceae*.

Phoma koolunga was not detected on native, naturalised or pasture legumes that had leaf spot symptoms, in any of the regions, and only one isolate was recovered from field pea. Analysis of DNA sequences from the ex-type culture of *P. koolunga* supported the change in nomenclature to *Ascochyta koolunga* **comb. nov.** Three new host-pathogen associations in Australia were *Didymella pinodes* on native *Senna artemisioides* as well as naturalised *Vicia cracca*; and *Didymella lethalis* on naturalised *Lathyrus tingitanus*.

Key words Alternative host, pathogen reservoir, multigene phylogeny

Introduction

The Didymellaceae was established to accommodate *Ascochyta, Didymella*, *Phoma* and other allied phoma-like genera (de Gruyter et al. 2009). The Didymellaceae now contains 19 genera (Ariyawansa et al. 2015, Aveskamp et al. 2010, Chen et al. 2015a, Chen et al. 2017, Crous and Groenewald 2016, Thambugala et al. 2017, Wijayawardene et al. 2016). Species of Didymellaceae are cosmopolitan and occupy a broad range of environments. Many species are plant pathogens that cause leaf and stem lesions, often with a broad host range (Aveskamp et al. 2009, Aveskamp et al. 2010, Chen et al. 2015b). DNA-based multilocus phylogenetics and a polyphasic approach to classify species have helped to revise taxa and refine systematic relationships in the Didymellaceae (Aveskamp et al. 2009, Aveskamp et al. 2010, Chen et al. 2015a, de Gruyter et al. 2009, de Gruyter 2012).

In Australia, reports of taxa in the Didymellaceae mostly refer to plant pathogenic species, particularly on crop and pasture legumes (Fabaceae). In Australia, the disease Ascochyta blight of *Pisum sativum* (field pea) is typically caused by up to three fungal species representing the genera *Didymella* and *Phoma*, namely, *Didymella pinodella, D. pinodes* and *Phoma koolunga*. A fourth species, *Ascochyta pisi*, is very rarely isolated. One species in particular, *Phoma koolunga*, is an important part of the Ascochyta blight disease complex of field pea in South Australia (Davidson et al. 2009a). First described in 2009, *P. koolunga* had spread across southern Australia and been detected in Victoria and Western Australia by 2015 (Davidson et al. 2011, Tran et al. 2015a).

Molecular techniques are now routinely used to understand the genetic diversity and population structure of *Ascochyta*, *Didymella* and *Phoma* (Aveskamp et al. 2010, Chen et al. 2015a, de Gruyter 2012, Hibbett et al. 2016, Salam et al. 2011a, Woudenberg et al. 2012). To date, there has not been a systematic inventory of leaf spot pathogens associated with

Australian native legume species despite international reports from a diversity of countries on Ascochyta blight since 2009 (Ahmed et al. 2015, Gaurilcikiene and Viciene 2013, Le May et al. 2009, Liu et al. 2013, Liu et al. 2016, Mathew et al. 2010, Panicker and Ramraj 2010, Skoglund et al. 2011, Soylu and Dervis 2011). *Phoma koolunga* is only known to occur in Australia, which suggests an Australasian origin, perhaps in association with native legume species. The aim of this study was to collect legume specimens from both cultivated and neighbouring natural ecosystems and to determine the species of Didymellaceae associated with leaf spot diseases. In particular, specimens were collected from Australian native, pasture and naturalised legumes in the field pea growing regions of eastern and southern Australia to investigate possible native sources of *P. koolunga.*

Materials and Methods

Sample collection and culture isolation

Samples of leaf spot diseases on legumes were obtained from 22 field pea trial sites, the immediate surrounds of experimental and commercial crops (see Appendix 3 Table I), and roadsides around crops in field pea growing regions of southern Australia. In total, 124 samples were collected during four separate 4−5 d periods in Aug, Sep and Oct 2017. In addition to trial sites, local agronomists were contacted to obtain approval to allow access to growers' properties in Eyre Peninsula (South Australia), and Horsham (Victoria).

The national parks, or conservation areas, nearest to the field pea sampling sites were identified prior to field trips and permits were obtained to enable collection of samples from native plants that exhibited leaf disease within these neighbouring natural ecosystems. Leaf disease samples were also collected from two botanic gardens, Adelaide, South Australia and Mount Annan, New South Wales. Plants with leaf spots were photographed in the field with a Samsung galaxy S5 or S8 mobile phone camera and the GPS locations recorded (see Appendix

3 Table II and Fig. I). Representative leaf samples were placed in plastic bags, labelled and stored at 4º C.

Within 5 d of collection leaf specimens were surface disinfected by spraying with 70 % v/v alcohol and blotted dry with fresh, non-sterilised tissue paper. Excised leaf pieces were placed on plates of potato dextrose agar (PDA) (Oxoid®) acidified by supplementation with 1 ml of 85 % v/v lactic acid per litre (APDA) to minimise bacterial contamination. Incubation was under a 12 h black and fluorescent light /12 h dark cycle at 22º C for 7−10 d, when fungal colonies were examined microscopically for pycnidia and conidia. Representative isolates were transferred by hyphal tip subculture to PDA and deposited in the culture collection of the Queensland Plant Pathology Herbarium (BRIP).

DNA extraction, PCR and sequencing

Genomic DNA was extracted from 7 d old mycelium grown on PDA from hyphal tip subcultures using the FastDNA® Kit (Q-biogene Inc. Irvine, California, USA) according to the manufacturer's instructions. A portion of DNA from the internal transcribed spacer (ITS) region was amplified with the primers ITS1 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 5′-TCCTCCGCTTATTGATATGC-3′ (White et al. 1990), and the partial region of the RNA polymerase II subunit B (*rpb*2) gene was amplified with the primers RPB2-5F2 5′-GGGGWGAYCAGAAGAAGGC-3′ (Sung et al. 2007) and RPB2-7cR 5′-CCCATRGCTTGYTTRCCCAT-3′ (Liu et al. 1999). The PCR conditions were as described by White et al. (1990) for ITS and O'Donnell et al. (2007) for *rpb*2. All PCR were amplified in 25 µl reaction volumes containing the final volumes; 1 of PCR buffer (Promega Corporation, Madison, Wisconsin, USA), 1.6 mM of 25 mM $MgCl_2$ (Sigma-Aldrich Corporation, Louis, Missouri, USA), 0.025 U/µL units of GoTaq™ (Promeg), 0.6 mM of primer 1 and primer 2 and 1.6 mM each of dATP, dCTP, dGTP and dTTP (Promega). The sequencing protocol being

followed was from the Ramaciotti Centre for Gene Function Analysis at the University of New South Wales (Randwick, NSW, Australia) and consists of four stages following PCR amplification; quantify RCR product on agarose gel, use ExoSAP-IT to clean PCR product, sequencing and purification (https://www.ramaciotti.unsw.edu.au/sites/default/files/2019-04/RAMAC_Sanger_Sequencing_Service_Guide_2019_v1.0.pdf). PCR products were electrophoresed on 2% agarose gel stained with ethidium bromide for visualisation under UV light to assess product integrity and estimate concentration. Removal of unused nucleotides using ExoSAP-IT (USB Corporation Cleveland, Ohio, USA) following the manufacturer's instructions was done if the PCR product was a pure single band.

The Sanger sequencing reaction was performed in a total volume of 20 µl consisting of; 0.34 µl sterile distilled H_2O, 3.5µl buffer, 1 µl big dye terminator, 0.16 µl of either forward or reverse primer (20 pmol) and 20-50 ng of PCR product using the same primers as for the PCR amplifications for each gene region, respectively. Both the forward and reverse strands were sequenced to minimise the presence of ambiguous nucleotides. Sequence run conditions were 10 s at 96º C followed by 5 s at 50º C then 4 mins at 60º C, repeated for 25 cycles. Samples were then purified using ethanol precipitation and dried before sealing and sending to the Ramaciotti Centre for Gene Function Analysis for DNA sequence determination using an ABI PRISM® 3700 DNA Analyser (Applied Biosystems Inc., Foster City, California, USA). PCR and sequencing were not repeated for any isolate if PCR and sequencing were successful at the first attempt. PCR was repeated once if there was no PCR product at the initial attempt for a limited number of isolates of interest, using previously prepared DNA. Replication of sequencing reactions to demonstrate reliable/repeatable results is not usual practice for sequence based molecular investigations; rather, quality and reliability are provided by the Phred score generated for each sequence (Dear and Staden, 1992).

Phylogenetic analysis

All sequences generated were assembled using Geneious v. 11.1.5 (Biomatters Ltd) and deposited in GenBank (Table 1, in bold). Sequences received included a Phred score, which provides an estimate of quality to aid in trimming of low-quality regions. Phred scores of >10 equate to > 90% base call accuracy. The sequences were trimmed for the expected poor quality at start due to poor primer binding and at end of sequences due to deterioration of sequence trace. Sequences were aligned with selected reference sequences of Didymellaceae obtained from GenBank using the multiple alignment MAFFT algorithm (Katoh et al. 2009) in Geneious. Reference sequences for each set of analyses (ITS and *rpb*2) were selected on the basis of the Basic Local Alignment Search Tool (BLAST) in GenBank to identify regions of similarity with previously published sequences, which would suggest a phylogenetic relationship within the genera in Didymellaceae (Chen et al. 2015a and 2017, Chilvers et al. 2009). Appropriate outgroups were determined using the results of *rpb*2 phylogenetic analyses. *Neoascochyta desmazieri* strain CBS 267.69 was chosen as a suitable outgroup for species resolution. The sequences of each locus were aligned separately and manually adjusted where necessary within the sequence alignment editing program in Geneious. Manual adjustment was performed to improve the alignment by identifying miss-alignments, errors in base call and investigation of repeated nucleotides, single-nucleotide polymorphisms (SNP) and conflict in base call between forward and reverse sequence were investigated and set to unknown (N) if appropriate. Gaps were treated as missing data.

Maximum likelihood (ML) analysis was run using RAxML v. 7.2.8 (Stamatakis and Alachiotis 2010) in Geneious and started from a random tree topology. The nucleotide substitution model used was general time-reversible (GTR) with a gamma-distributed rate variation. Clade stability was assessed in Geneious using 1,000 heuristic search bootstrap replications with random sequence addition. The Bayesian analysis was performed using

MrBayes v.3.2.1 (Ronquist and Huelsenbeck 2003) in Geneious. To remove the need for *a priori* model testing, the Markov chain Monte Carlo (MCMC) analysis was set to sample across the entire GTR model space with a gamma-distributed rate variation across the sites. Ten million random trees were generated using the MCMC procedure with four chains. The sample frequency was set at 100 and the temperature of the heated chain was 0.1. "Burn-in" was set at 25 %, after which the likelihood values were stationary.

Morphology

Three replicate plates of each fungal isolate were grown on four media; PDA, oat agar (OA), malt extract agar (MEA) (Boerema et al. 2004, Chen et al. 2015a, Davidson 2009). To induce the formation of the asexual morph in culture, appropriate techniques use sterilized pieces of plant materials (Hyde et al. 2016). Carnation leaf agar (CLA) was chosen for this study due to a readily available supply of irradiated carnation leaves. Colony characters were observed and recorded. The colonies were measured (diameter) at 7 d and again after 12-14 d incubation, described and morphology examined, including mycelial colour, shape of colony margin, variations in mycelial patterns and pigmentation of agar, front and reverse of plates. Images of the colonies were captured by an Epson Perfection V700 scanner at a 300 dpi resolution. Colony colour was determined on surface and reverse using the colour charts of Rayner (1970). Isolates were characterised microscopically from PDA plates. Lactic acid (100 % v/v) was used as the mounting fluid. Specimens were examined using a Leica DM5500B compound microscope with a Leica DFC 500 camera fitted to capture images under Nomarski differential interference contrast illumination. Micromorphological measurements and descriptions of pycnidia, pycnidial wall cells and conidia were taken from up to 20 samples, and septation and colour recorded. Images of pycnidia were taken from CLA plates using a Leica M165C stereo microscope and Lecia DFC 500 camera. The NaOH spot test on one MEA culture plate per

isolate was used as it can help distinguish taxa in Didymellaceae (Chen et al. 2017). Some species of *Phoma* produce a colourless diffusible antibiotic metabolite 'E' (after *Phoma exigua*). One drop of concentrated (1N) NaOH on MEA plates is used to reveal if this metabolite oxidizes into the pigments 'α' and 'β'. Pigment α is red-purple at pH < 10.5 and blue-green at pH > 12.5. Pigment β is yellow at pH < 3.5 and red at pH > 5.5. Metabolite E-producing cultures react within about 10 min to produce a greenish spot or ring (pigment α), which changes to red (pigment β) after about 1 h (Boerema et al. 2004).

Results

From 124 samples of legumes collected at 22 locations, 194 isolates were obtained of which 54 isolates were identified as Didymellaceae by ITS sequences. Of these, 36 isolates were further sequenced (*rpb*2 locus). Within the 36 isolates there were 18 isolates belonging to either *P. sativum,* where the *rpb*2 sequence was shown to be a 100% match for *D. pinodes* or *D. pinodella,* or belonged to *V. sativa* where the sequence was shown to be *Ascochyta medicangicola,* all of which are expected pathogens on these hosts. Isolates of these pathogens on these hosts were not the focus of this research so were removed from analysis. Only one isolate of *D. pinodes* and *D. pinodella* from *P. sativum* were included in the remaining 18 isolates. The remaining 18 isolates were used for multilocus sequence analysis and representation in phylogenetic trees.

Phylogeny

A multilocus sequence analysis based on the ITS region and partial region of the *rpb*2 gene was used to infer the relationship of the 18 isolates and recognised species in Didymellaceae (Table 1). The resulting concatenated aligned dataset comprised 83 ingroup isolates from 74 taxa, and consisted of 1,101 characters (497 for ITS, and 604 for *rpb*2, including alignment

gaps). The ML tree based on the combined dataset is presented, with bootstrap support values (BS) greater than 70% and Bayesian posterior probabilities (PP) greater than 0.7 indicating four well-supported clades (Fig. 1, also see Appendix 3, Fig. II). The ITS phylogeny, using either ML or Bayesian analysis, provided poor resolution at the genus and species level (see Appendix 3, Fig. III). This is likely to be because of lack of divergence and taxon sampling density, however the ITS gene serves as a primary barcode locus as it is easily generated and has the most extensive dataset available (Quaedvlieg et al. 2014), consequently it was used in this research. The phylogenetic tree based on the concatenated alignment of ITS and *rpb*2 (Fig. 1) resolved the 18 isolates into ten species from Didymellaceae represented by; two isolates belonging to *Ascochyta,* eight *Didymella,* seven *Nothophoma* and one *Neodidymelliopsis*.

Phylogenetic analysis identified among the field collections three new host-pathogen associations for Australia (Fig. 1 and see Appendix 3, Table III). *Didymella pinodes* was isolated from native *S. artemisioides* from five locations in SA separated by over 400 km. Three locations were close by field pea crops (example, BRIP 69596, Fig. 8). Two locations were highway roadsides, near Blanchetown in the Riverina and Murray River regions, which are not field pea growing areas. *Didymella pinodes* was also isolated from naturalised *Vicia cracca* (tufted vetch) in NSW from a non-field pea growing area (BRIP 69578, Fig. 9). *Didymella lethalis* was isolated from the naturalised *Lathyrus tingitanus* (tangier pea) from a recreation walking area within an urban environment (BRIP 69584, Fig. 10). The isolates of *D. lethalis* and *D. pinodes* from *S. artemisioides* were morphologically similar with respect to colony colour, scalloped shape at colony margins, size and shape and size of pycnidia and conidia (Figs 8 and 10, also see descriptions in Appendix 3).

From the phylogenetic analysis of the *Ascochyta* isolates, one new combination is proposed. and is described below in the taxonomy section of results. Justification for placement of *Ascochyta koolunga* is based on phylogenetic analysis showing that it fits into that genus with

100% identity with ex-holotype of *Ascochyta boeremae* and holotype *Phoma koolunga*. It is difficult to identify which node in the tree corresponds to which SNPs in the alignment that isolates share to place it in *Ascochyta* rather than *Phoma*. Looking for the part of a tree that is under heavy selection pressure leading to development of a pathogenic species is not usually attempted as the methods are not fully developed. The phylogenetic tree just provides strong support for the placement. The reported morphology of these two taxa differs in conidium size, but both are described as highly variable. *Conidia* mostly aseptate, 12.5–17 × 5–7 µm for *P. koolunga,* compared with 16.5–26 × 4.5–7.5 µm for *A. boeremae*. Growth on both OA and MEA after 7 d was greater for *A. boeremae,* with colony diameters 25–30 mm and 20–25 mm respectively, compared with 13–20 mm and 7–12 mm for *P. koolunga.* The colony colours were similar (Chen et al. 2017; Davidson et al. 2009). Differences could be attributable to culture media used and incubation temperature, which differed by 3º C, 22º C for *P. koolunga* compared with 25º C for *A. boeremae*. Several isolates of each species need to be compared directly using standardised media and growth conditions to confirm to proposed combination and re-naming.

Taxonomy

Multi-locus sequence analysis and morphological comparisons classified ten fungal isolates from legumes in southern Australia into six novel species belonging to three genera of Didymellaceae. The novel species are described and illustrated in Figs 2-7. Nomenclatural novelties are registered in MycoBank.

The species epithets were derived from Indigenous Australian Peoples' language groups to provide a uniquely Australian theme. Permission to use words from the local language of the area in which the fungi were collected was granted by elders or community representatives (see Appendix 2).

Ascochyta koolunga **(J.A. Davidson, D. Hartley, M. Priest, M. Krysinka-Kaczmarek, Herdina, A. McKay & E.S. Scott) E.C. Keirnan, R.G. Shivas & Y.P. Tan, comb. nov.**

MycoBank MB833688

Basionym. *Phoma koolunga* J.A. Davidson, D. Hartley, M. Priest, M. Krysinka-Kaczmarek, Herdina, A. McKay & E.S. Scott, Mycologia 101(1): 120. 2009.

= *Ascochyta boeremae* L.W. Hou, P. W. Crous & L. Cai, Studies in Mycology. 87: 126. 2017.

Description. (Davidson et al. 2009)

Specimens examined. AUSTRALIA, South Australia, Minnipa, from stem of *Pisum sativum,* 26 Oct 2004*, J.A. Davidson* (holotype of *P. koolunga* DAR 78535, includes culture ex-type); Riverton, *P. sativum,* 2015, *J.A. Davidson* (BRIP 70265); Mundulla, *P. sativum,* 27 Aug 2017, *E.C. Keirnan* (BRIP 69590); from leaf of *P. sativum*, deposited in CBS Sep. 1984, *G.H. Boerema* (holotype of *A. boeremae* CBS H-23017, ex-holotype culture CBS 372.84 = PD 80/1246); from leaf of *P. sativum*, deposited in CBS Sep. 1984, *G.H. Boerema* (paratype of *A. boeremae* CBS H-9078, culture CBS 373.84 = PD 80/1247).

Notes. This proposed new combination is supported by the phylogenetic tree inferred from the combined multilocus alignment, which shows that the ex-holotype isolate of *P. koolunga* (DAR 78535) and the ex-holotype isolate of *A. boeremae* (CBS 372.84) are identical (Fig. 2). *Ascochyta boeremae* was the name given to a new species represented by two cultures (CBS 372.84 and CBS 373.84) isolated from *P. sativum* by G.H. Boerema, *circa* 1984 in South Australia (Chen et al. 2017).

Ascochyta koolunga is one of a complex of closely related species, which individually or collectively cause the destructive disease known as Ascochyta blight of field pea, known since the 1960s (Ali and Dennis 1992) in regions with Mediterranean climates in Australia (Davidson

et al. 2009, Davidson et al. 2011, Tran et al. 2015a). The proportional percentage infection rates of field pea in South Australia were reported as *A. koolunga* 41 %; *Didymella pinodes* 54 %; and *D. pinodella* 5 % (Davidson et al. 2009). During this present study, *A. koolunga* was not isolated from native, naturalised or pasture legumes that had leaf spot symptoms, and only one isolate was recovered from field pea. Worldwide, the genus *Ascochyta* is known to include several species that show relatively high host specificity to Fabaceae (Chen et al. 2017). For additional confirmation of the re-naming as *Ascochyta* and proposed new combination/correction to nomenclature, the two isolates of *P. koolunga* used in this research listed in Table 1 have subsequently been sequenced at the additional loci, *tub*2 and LSU, as used by Chen et al. (2017) to enable the re-naming of *A. boeremae.* The sequences were 100 % identical (data not shown). Complete genome sequences could be obtained and compared to examine similarity and, in doing so, confirm or refute the relationship proposed.

Didymella djirangnandiri **E.C. Keirnan, M.H. Laurence, R.G. Shivas & Y.P. Tan, sp. nov.**

MycoBank MB833689

Fig. 2

Type. AUSTRALIA, New South Wales, Mount Annan, *Swainsona galegifolia*, 19 Jan. 2017, *E.C. Keirnan* (holotype BRIP 69585, includes culture ex-type).

Description. *Colonies* on OA, 76–80 mm diam. after 7 d, covered in dense aerial mycelium, variable shades of grey, pale cinnamon towards centre; reverse dark vinaceous; on MEA, 70–72 mm after 7 d, margin entire, covered in low dense aerial mycelium, pale mouse grey with lighter patches; reverse olivaceous with radiating spokes; on PDA, 73–80 mm after 7 d, margin entire, mycelia felty, mouse grey becoming vinaceous buff towards centre; reverse fuscous black. *NaOH spot test*: negative. *Conidiomata* on CLA, pycnidial, globose 100–200 µm diam.,

pale brown becoming black, solitary, glabrous, non-papillate; pycnidial wall composed of textura globulosa, pale brown, cells 5–15 µm diam. *Conidiogenous* cells phialidic, cylindral, thin-walled, hyaline, rounded ends. *Conidia* aseptate, 5–7 × 2–3 µm.

Etymology. From the language of the Indigenous Australian Dharawal people, meaning leaf spot. The Dharawal people are from the western Sydney region in New South Wales, which includes Mount Annan, where the holotype was collected.

Notes. *Didymella djirangnandiri* is sister to the clade containing other *Didymella* species (Fig. 2). *Didymella djirangnandiri* has small aseptate conidia that are mostly guttulate, which is similar to most other *Didymella* species. *Didymella djirangnandiri* is only known from one specimen on *Swainsona galegifolia*. The sister status requires review with other taxa included from references sequences in a re-analysis to confirm genus *Didymella.*

Didymella kaurna **E.C. Keirnan, M.H. Laurence, R.G. Shivas & Y.P. Tan, sp. nov.**

MycoBank MB833690

Fig. 3

Type. AUSTRALIA, South Australia, Adelaide, *Gastrolobium celsianum*, 17 Sep. 2017, *E.C. Keirnan* (holotype BRIP 69579, includes culture ex-type).

Description. *Colonies* on OA, 40–45 mm diam. after 7 d, margins entire, flat with abundant patches of dense pale mouse grey aerial mycelium, buff with abundant vinaceous grey pycnidia; reverse buff with concentric rings of vinaceous grey pycnidia with ochreous patches; on MEA, 37–40 mm after 7 d, margin entire, flat, buff with abundant brown vinaceous pycnidia in concentric rings denser towards the centre; reverse buff with dark zonate rings; on PDA, 40–45 mm after 7 d, margin entire, irregular dense pale vinaceous buff aerial mycelium central part with abundant dark pycnidia; reverse buff darker at centre. *NaOH spot test*: negative.

Conidiomata on CLA, pycnidial, globose to subglobose, 130–320 µm diam., pale brown, scattered, abundant, zonate, glabrous, non-papillate; ostiole c. 25 µm diam.; pycnidial wall composed of textura angularus, pale to medium brown, cells 5–12 µm diam. *Conidiogenous* cells phialidic, cylindrical, thin-walled, hyaline 5–12 × 2–4 µm long, narrower at the apex. *Conidia* aseptate, 5–7.0 × 2.0–3.0 µm, parallel to narrowly ellipsoidal, hyaline, wall c. 0.5 µm.

Etymology. The Indigenous Kaurna people are from the Adelaide plains region, which includes central Adelaide, the locality from where the holotype was collected.

Notes. *Didymella kaurna* was phylogenetically closest to the ex-type culture of *D. sinensis* (strain CGMCC 3.18348) with one nucleotide difference in a blast search (Chen et al. 2017). However, the *rpb*2 sequence of *D. sinensis* strain CGMCC 3.18348 was not available for further phylogenetic comparison. An isolate identified as *D. sinensis* (strain LC 5246) differed from *D. kaurna* in *rpb*2 (with 94 % identity) (Chen et al. 2017). As *D. sinensis* was only observed as a sexual morph (Chen et al. 2017), a morphological comparison could not be made with *D. kaurna*. *Didymella kaurna* did not produce a reaction in the NaOH spot test, which distinguished it from *D. sinensis* (Chen et al. 2017).

Neodidymelliopsis tinkykuku **E.C. Keirnan, M.H. Laurence, R.G. Shivas & Y.P. Tan, sp. nov.**

MycoBank MB833692

Fig. 4

Type. AUSTRALIA, South Australia, Clare, *Hardenbergia violacea*, 17 Sep. 2017, *E.C. Keirnan* (holotype BRIP 69592, includes culture ex-type).

Description. *Colonies* on OA, 26–28 mm diam. after 7 d, dense low aerial mycelium, buff with numerous grey patches, darker with abundant pycnidia at centre; reverse buff to rosy buff with darker concentric rings towards centre; on MEA, 28–30 mm after 7 d, margin entire, dense low aerial mycelium, vinaceous buff paler at margin; reverse rosy buff to buff at margin with abundant scattered pycnidia; on PDA, 35–38 mm after 7 d, margin entire, dense low aerial mycelium, pale mouse grey lighter at margin; reverse cinnamon with concentric dark rings, darker at centre. *NaOH spot test:* light yellow. *Conidiomata* on CLA pycnidial, globose to ampulliform, 250–350 µm diam., brown becoming black, solitary, abundant in centre of colony, zonate, glabrous, non-papillate; ostiole c. 25 µm diam.; pycnidial wall composed of textura angularus, pale brown, cells 5–8 µm diam. *Conidiogenous cells* phialidic, cylindrical, thin-walled, hyaline. *Conidia* occasionally septate, 6–9 × 2–3 µm, cylindrical, hyaline, thin-walled.

Etymology. From the language of the Indigenous Australian Kaurna people, meaning leaf disease. The Kaurna people are from the Adelaide plains region, which includes Clare, the locality where the holotype was collected.

Notes. *Neodidymelliopsis tinkyukuku* (strain BRIP 69592) is phylogenetically close to the ex-type culture of *N. ranunculi* (strain MFLUCC 13-0490) with 99 % identity (462/465 nucleotides) in the ITS region. The *rpb*2 sequence of *N. ranunculi* strain MFLUCC 13-0490 was not available for further phylogenetic comparison. *Neodidymelliopsis tinkyukuku* was also sister to *N. farokhinejadii* and is easily distinguished by *rpb*2 sequences (96 % identity).

Nothophoma garlbiwalawarda **E.C. Keirnan, M.H. Laurence, R.G. Shivas & Y.P. Tan, sp. nov.**

MycoBank MB833693

Fig. 5

Type. AUSTRALIA, South Australia, Wudinna, *Senna artemisioides*, 19 Aug. 2017, *E.C. Keirnan* (holotype BRIP 69595, includes culture ex-type).

Description. *Colonies* on OA, 27–30 mm diam. after 7 d, flat with scant aerial mycelia with a few zonate rings, vinaceous to dark vinaceous; vinaceous to dark vinaceous; on MEA, 23–25 mm after 7 d, margin entire, flat, scant aerial mycelium towards centre, amber with abundant pycnidia; reverse amber darker towards centre; on PDA, 28–30 mm after 7 d, margin irregular, flat with aerial mycelia tufted in centre, dark with abundant pycnidia in concentric rings, buff at margin; reverse dark becoming buff at margin. *NaOH spot test*: reddish. *Conidiomata* pycnidial, globose to subglobose, 130–320 µm diam., pale brown, scattered, abundant, zonate, glabrous, non-papillate; ostiole c. 25 µm diam.; pycnidial wall composed of textura angularus, pale to medium brown, cells 5–12 µm diam. *Conidiogenous* cells phialidic, cylindrical, thin-walled, hyaline 5–12 × 2–4 µm long, narrower at the apex. *Conidia* aseptate, 5–7.0 × 2.0–3.0 µm, parallel to narrowly ellipsoidal, hyaline, wall c. 0.5 µm.

Etymology. From the native language of the Indigenous Australian Barngarla people, meaning leaf-fun-guy. The Barngarla people are from the Eyre Peninsula region, which includes Wudinna, the locality where the holotype was collected.

Additional material examined. AUSTRALIA, South Australia, Adelaide, *Senna artemisioides*, 26 Oct. 2016, *E.C. Keirnan* (BRIP 69580), (see Appendix 3, Fig IV); Berri, *Senna artemisioides*, 01 Jul. 2017, *E.C. Keirnan* (BRIP 69586), (see Appendix 3 Fig. V); ibid, 01 Jul. 2017, *E.C. Keirnan* (BRIP 69587), (see Appendix 3 Fig. VI); Kimba, *Senna artemisioides*, 17 Sep. 2017, *E.C. Keirnan* (BRIP 69594).

Notes. *Nothophoma garlbiwalawarda* is phylogenetically closest to *Nothophoma anigozanthi* (Fig. 2). *Nothophoma garlbiwalawarda* is distinguished from *No*. *anigozanthi* by *rpb*2 sequence (93 % identity). The conidia of *No. garlbiwalawarda* are larger than those of *No. anigozanthi* (3.5–5 × 1.5–2.5 µm). The *NaOH spot test* of *No. anigozanthi* produced on MEA

a luteous discolouration later changing to dull green then black, which is distinguished from *No. garlbiwalawarda* which produced a reddish reaction.

Nothophoma naiawu **E.C. Keirnan, M.H. Laurence, R.G. Shivas & Y.P. Tan, sp. nov.**

MycoBank MB833694

Fig. 6

Type. AUSTRALIA, South Australia, Blanchetown, from *Senna artemisioides*, 22 Oct. 2016, *E.C. Keirnan*, holotype BRIP 69583 (includes culture ex-type).

Description. *Colonies* on OA, 21–25 mm diam. after 7 d, flat with scant aerial mycelia, rosy vinaceous, dark at centre; reverse rosy buff, dark at centre, with a few dark radiating fissures; on MEA, 27–30 mm after 7 d, margin entire, flat, with sparse aerial mycelium towards centre rosy vinaceous; reverse peach, darker at centre; on PDA, 27–30 mm after 7 d, margin entire, flat felty, rosy buff; reverse peach, dark at centre. *NaOH spot test:* slightly yellow. *Conidiomata* pycnidial, globose to subglobose, 200–300 μm diam., pale brown becoming black, semi-immersed, confluent on MEA, glabrous, non-papillate; ostiole c. 25 μm diam.; pycnidial wall composed of textura globulosa, pale brown, cells 5–8 μm diam.. *Conidiogenous* cells phialidic, cylindrical, very thin-walled, hyaline. *Conidia* aseptate or 1-septate, 8–12 × 4–6 μm, cylindrical to narrow ellipsoidal, pale yellow.

Etymology. A variation of the Indigenous Australian Ngayawang people's language group, who lived in the Murray River region of South Australia, which includes Blanchetown, the locality where this specimen was collected.

Notes. *Nothophoma naiawu* is phylogenetically close to *No. arachidis-hypogaeae* (Fig. 2). *Nothophoma naiawu* is distinguished from *No. arachidis-hypogaeae* by the ITS region (98 % identity) and the *rpb*2 locus (98% identities). Morphologically, the conidia of *No. naiawu* are

aseptate or 1–septate, 8–12 × 4–6 µm and pale yellow, whereas conidia of *No. arachidis-hypogaeae* are much smaller, 3.2–5.2 × 1.8–2.4 µm.

Nothophoma ngayawang **E.C. Keirnan, M.H. Laurence, R.G. Shivas & Y.P. Tan, sp. nov.**

MycoBank MB833695

Fig. 7

Type. AUSTRALIA, South Australia, Blanchetown, *Senna artemisioides*, 22 Oct. 2016, *E.C. Keirnan*, holotype BRIP 69582 (includes culture ex-type).

Description. *Colonies* on OA, 18–20 mm diam. after 7 d, covered by scant tufted aerial mycelia at centre becoming abundant and floccose towards margin, rosy buff becoming darker towards centre; reverse salmon with centre and margins pale isabelline; on MEA, 15–20 mm after 7 d, margin irregular, felty buff becoming white towards the margin; reverse pale rosy buff, darker at centre becoming paler near margin; on PDA, 18–21 mm after 7 d, margin regular, aerial mycelia tufted in centre becoming floccose toward the margin, white to pale rosy buff; reverse pale rosy buff with few scattered vinaceous spots. *NaOH spot test*: slightly yellow. *Conidiomata* pycnidial, globose to subglobose, 200–300 µm diam., pale brown becoming black, solitary, abundant in centre of colony, glabrous, non-papillate; ostiole c. 25 µm diam.; pycnidial wall composed of textura globulosa, pale brown, cells 5–8 µm diam. *Conidiogenous* cells phialidic, cylindrical, thin-walled, hyaline. *Conidia* aseptate, 2.5–4.0 × 1.0–2.0 µm, cylindrical to narrow ellipsoidal, hyaline, thin-walled.

Etymology. Named after the Indigenous Australian Ngayawang people's language group, who existed in the Murray River region of South Australia, which includes Blanchetown, the locality where this specimen was collected.

Notes. *Nothophoma ngayawang* is phylogenetically close to *No. variabilis* (Fig. 2). *Nothophoma ngayawang* is distinguished from *No. variabilis* by the ITS region (98 % identity) and the *rpb*2 locus (93% identities). Both species have small non-septate conidia, and the conidiogenous cells are similar in colour, size and shape (Valenzuela-Lopez et al. 2018). The *NaOH spot test* of *No. variabilis* was negative on MEA, which is distinguished from the slightly yellow reaction of *No. ngayawang*.

Discussion

Our phylogenetic investigations of novel Didymellaceae species revealed strong phylogenetic evidence that *P. koolunga* should be re-named as *Ascochyta koolunga* because there is a species synonymy with *A. boeremae*. *Ascochyta koolunga* was not identified on native Australian legumes in this study. The incidence of *A. koolunga* in Ascochyta blight-affected crops was low in southern Australia, with only one isolate (BRIP 69590) from field pea collected from Mundulla, SA. The incidence of *A. koolunga* varies from year to year, with low incidence reported during Ascochyta blight disease monitoring in 2017 (L. McMurray, South Australian Research and Development Institute, *pers. comm.*). It is difficult to make an association between the low incidence of *A. koolunga* in field pea and the absence of *A. koolunga* on other legumes. Evidence to date suggests it is unlikely that *A. koolunga* originated from Australia native legumes because *A. koolunga* was not collected from any of the native legume species among the samples collected in this thesis study. Further research is required to test this suggestion.

Six novel species of Didymellaceae were instead found in this study. Two *Didymella* species, *D. djirangnandiri* (on *S. galegifolia*) and *D. kaurna* (on *G. celsianum*), were collected from plants in the botanic gardens in New South Wales and South Australia, respectively. *Gastrolobium celsianum* is endemic to southwest Western Australia, not Adelaide, where the

specimen was collected. *Swainsonia galegifolia* is endemic to east coast New South Wales where the specimen was collected. *Neodidymelliopsis tinkyukuku* from *H. violacea* was collected from a tourist bureau car park garden, growing adjacent to *V. sativa* (common vetch) from which *D. pinodes*, the primary Ascochyta blight pathogen, was isolated. *Hardenbergia violacea* has a wide distribution in southern and eastern Australia, including South Australia, where the specimen was collected. These three native Australian legume species were cultivated, possibly from nursery-generated seedlings or cuttings, and not in a natural environment. Further studies are warranted to understand how widespread these fungal species may be in cultivated or natural environments, and if they are host specific. Two years post-isolation of *D. kaurna* from one *G. celsianum* plant, that plant had died, whilst a neighbouring plant was relatively healthy, which raises questions about the epidemiology, life cycle, and pathogenicity of this species.

Leaf spots were commonly seen on the native legume *S. artemisioides* throughout all regions visited in South Australia. Three novel *Nothophoma* species were isolated from *S. artemisioides*. *Nothophoma garlbiwalawarda* was collected from five locations across South Australia, separated by over 400 km, in field pea and non-field pea growing regions. The isolate from Wudinna, Eyre Peninsula was selected as the type species of *No. garlbiwalawarda.* There was some morphological difference between isolates in terms of conidial size and colour, but phylogenetically the five isolates were identical. *Nothophoma naiawu* and *No. ngayawang* were isolated from *S. artemisioides* collected from the South Australian Murray River region along a main highway roadside. The leaf spot symptoms for all three *Nothophoma* species were small pin-prick lesions, with some larger spots on seed pods caused by *No. ngayawang.* The species were indistinguishable based on symptoms alone.

None of the symptomatic legume leaf samples collected originated from national parks or conservation areas. Most of the legumes in natural ecosystems looked healthy, with few or no

leaf spots. A disadvantage in several national parks was a general lack of legume species, whether lower canopy shrubs, prostrate or climbing varieties. Additionally, collection was not possible at one park in New South Wales due to a recent bush fire event, which had burnt out the understorey, eliminating any visible legume species from the area. All isolates of interest came from legumes collected in cultivated environments with varying degrees of human disturbance, and thus not considered a natural environment. However, a rigorous and systematic longitudinal biodiversity survey could identify additional novel leaf pathogens, or existing pathogens from more new hosts.

The new host-pathogen associations found in the field collections included *D. pinodes*, the primary Ascochyta blight pathogen, from naturalised *Vicia cracca* (tufted vetch). This host could certainly be a reservoir of Ascochyta blight inoculum if growing adjacent to field pea crops. This discovery of an alternative host has implications for disease epidemiology and management. The second new host for *D. pinodes* was *S. artemisioides* and the symptoms are indistinguishable from the pin-prick leaf spot symptoms caused by the novel *Nothophoma* species. *Didymella pinodes* was isolated from five locations. Four of these locations also yielded a novel *Nothophoma* species. The size of the lesions on *S. artemisioides* would suggest a hypersensitive reaction to *D. pinodes* and perhaps it would not be a meaningful alternative inoculum source for this Ascochyta blight pathogen. The third new host-pathogen association for Australia was *D. lethalis* from *Lathyrus tingitanus* (tangier pea). *Didymella lethalis* is considered closely related to *D. pinodes* based on phylogenetic data, with clarification of the relationship between the two species reported as awaiting examination of the type specimen (Chen et al. 2015a).

This study uncovered six novel isolates in the Didymellaceae from Australian native legumes, and identified three new legume host-pathogen associations for Australia. *Ascochyta koolunga* was not isolated from species other that field pea, which might be an artefact of the

low incidence of the fungus *A. koolunga* during the collection period. Further investigations using a longitudinal systematic survey are needed to identify any native hosts of *A. koolunga* and to further investigate the diversity and prevalence of Didymellaceae species on Australian native, pasture and naturalised legumes, to classify novel isolates and to identify new Australian hosts for known species.

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Table 1. Didymellaceae isolates examined in this study. Novel taxa and newly generated sequences are indicated in **bold**.

Species	Strain [1]	Host	Locality [2]	GenBank accessions [3]	
				ITS	***rpb2***
Ascochyta fabae	CBS 524.77	*Phaseolus vulgaris*	Belgium	GU237880	
Ascochyta herbicola	CBS 629.97	Water	USA, Montana, Missoula	GU237898	KP330421
***Ascochyta koolunga* comb. nov.**	DAR 78535 [T]	*Pisum sativum*	Australia, SA, Minnipa	EU338416	EU874849
	BRIP 70265	*Pisum sativum*	Australia, SA, Riverton	**MN567671**	**MN604922**
	BRIP 69590	*Pisum sativum*	Australia, SA, Mundulla	**MN567672**	**MN604923**
	CBS 372.84 [T]	*Pisum sativum*	Australia, SA	KT389480	
	CBS 373.84	*Pisum sativum*	Australia, SA	KT389481	KT389560
Ascochyta lentis	CBS 370.84	*Lens culinaris*	Unknown	KT389474	
Ascochyta medicaginicola var. *macrospora*	BRIP 45051	*Medicago sativa*	Australia, NSW, Wagga Wagga	KY742044	KY742132
	CBS 404.65	*Medicago sativa*	Canada, Saskatchewan, Saskatoon	GU237859	KP330423
Ascochyta medicaginicola var. *medicaginicola*	CBS 316.90	*Medicago sativa*	Czech Republic	GU237828	
Ascochyta nigripycnidia	CBS 116.96 [T]	*Vicia cracca*	Russia	GU237756	
Ascochyta phacae	CBS 184.55 [T]	*Phaca alpina*	Switzerland	KT389475	
Ascochyta pisi	CBS 126.54	*Pisum sativum*	The Netherlands	GU237772	DQ677967
	CBS 122751	*Pisum sativum*	Canada, Saskatchewan, Saskatoon	KP330432	EU874867
Ascochyta rabiei	CBS 237.37 [T]	*Cicer arietinum*	Bulgaria	KT389479	
	CBS 534.65	*Cicer arietinum*	India	GU237886	KP330405
	CBS 206.30	*Cicer arietinum*	Unknown	KT389478	KT389559
Ascochyta syringae	CBS 545.72	*Syringa vulgaris*	The Netherlands	KT389483	
Ascochyta versabilis	CBS 876.97	*Silene* sp.	The Netherlands, Wageningen	GU237909	KT389561
Ascochyta viciae	CBS 451.68	*Vicia sepium*	The Netherlands, Baarn, Praamgracht	KT389484	KT389562
Ascochyta viciae-pannonicae	CBS 254.92	*Vicia pannonica*	Czech Republic	KT389485	
Didymella aeria	CGMCC 3.18353 [T]	Air	China	KY742051	KY742137
Didymella aliena	CBS 379.93	*Berberis* sp.	The Netherlands	GU237851	KP330416
Didymella americana	CBS 185.85	*Zea mays*	USA, Georgia	FJ426972	KT389594
Didymella anserina	CBS 360.84	Potato flour	The Netherlands	GU237839	KT389596
Didymella aquatica	CGMCC 3.18349 [T]	Water	China	KY742055	KY742140
Didymella arachidicola	CBS 333 .75 [T]	*Arachis hypogaea*	South Africa, Cape Province	GU237833	KT389598
Didymella aurea	CBS 269.93 [T]	*Medicago polymorpha*	New Zealand, Auckland	GU237818	KT389599

Didymella bellidis	CBS 714.85	*Bellis perennis*	The Netherlands	GU237904	KP330417
Didymella boeremae	CBS 109942 [T]	*Medicago littoralis* cv. Harbinger	Australia, VIC	FJ426982	KT389600
Didymella brunneospora	CBS 115.58 [T]	*Chrysanthemum roseum*	Germany, Berlin	KT389505	KT389625
Didymella chenopodii	CBS 128.93	*Chenopodium quinoa* cv. Sajana	Peru	GU237775	KT389602
Didymella chloroguttulata	CGMCC 3.18351 [T]	Air	China	KY742057	KY742142
Didymella coffeae-arabicae	CBS 123380 [T]	*Coffea arabica*	Ethiopia	FJ426993	KT389603
Didymella curtisii	PD 92/1460	*Sprekelia* sp.	The Netherlands	FJ427041	KT389604
***Didymella djirangnandiri* sp. nov.**	BRIP 69585 [T]	*Swainsona galegifolia*	Australia, NSW, Mount Annan	**MN567673**	**MN604924**
Didymella ellipsoidea	CGMCC 3.18350 [T]	Air	China	KY742060	KY742145
Didymella eucalyptica	CBS 377.91	*Eucalyptus* sp.	Australia, Western Australia (WA)	GU237846	KT389605
Didymella exigua	CBS 183.55 [T]	*Rumex arifolius*	France, Menise sur Tholon	GU237794	EU874850
Didymella glomerata	CBS 528.66	*Chrysanthemum* sp.	The Netherlands	FJ427013	GU371781
Didymella heteroderae	CBS 109.92 [T]	Undefined food material	The Netherlands	FJ426983	KT389601
Didymella ilicicola	CGMCC 3.18355 [T]	*Ilex chinensis*	Italy	KY742065	KY742150
Didymella infuscatispora	CGMCC 3.18356 [T]	*Chrysanthemum indicum*	China	KY742067	KY742152
Didymella keratinophila	UTHSC DI16-200 [T]	*Homo sapiens*	USA	LT592901	LT593039
Didymella lethalis	CBS 103.25	Unknown	Unknown	GU237729	KT389607
	BRIP 69584	*Lathyrus tingitanus*	Australia, SA, Brownhill Creek	**MN567674**	**MN604925**
Didymella macrophylla	CGMCC 3.18357 [T]	*Hydrangea macrophylla*	Italy	KY742070	KY742154
Didymella macrostoma	CBS 223.69	*Acer pseudoplatanus*	Switzerland	GU237801	KT389608
Didymella maydis	CBS 588.96 [T]	*Zea mays*	USA, Wisconsin, Hancock	FJ427086	GU371782
Didymella microchlamydospora	CBS 105.95 [T]	*Eucalyptus* sp.	UK	FJ427028	KP330424
Didymella negriana	CBS 358.71	*Vitis vinifera*	Germany, Oberdollendorf am Rhein	GU237838	KT389610
Didymella nigricans	LC8134	*Acer palmatum*	Japan	KY742075	KY742158
Didymella pedeiae	CBS 124517 [T]	*Schefflera elegantissima*	The Netherlands	GU237770	KT389612
Didymella pinodella	CBS 531.66	*Trifolium pratense*	USA, Minnesota	FJ427052	KT389613
	BRIP 69589	*Pisum sativum*	Australia, VIC, Rainbow	**MN567675**	**MN604926**
Didymella pinodes	CBS 525.77 [T]	*Pisum sativum*	Belgium	GU237883	KT389614
	BRIP 69581	*Senna artemisioides*	Australia, SA, Blanchetown	**MN567676**	**MN604927**
	BRIP 69593	*Senna artemisioides*	Australia, SA, Blyth	**MN567677**	**MN604928**
	BRIP 69596	*Senna artemisioides*	Australia, SA, Wudinna	**MN567678**	**MN604929**
	BRIP 69578	*Vicia cracca*	Australia, NSW, Cowra	**MN567679**	**MN604930**
Didymella pomorum	CBS 539.66	*Polygonum tataricum*	The Netherlands	FJ427056	KT389618
Didymella protuberans	CBS 381.96 [T]	*Lycium halifolium*	The Netherlands	GU237853	KT389620

Didymella pteridis	CBS 379.96 ^{T}	*Pteris* sp.	The Netherlands	KT389504	KT389624
Didymella rhei	BRIP 5562	*Rheum rhaponticum*	Australia	KY742083	KY742163
	CBS 109177	*Rheum rhaponticum*	New Zealand	GU237743	KP330428
Didymella rumicicola	CBS 683.79 ^{T}	*Rumex obtusifolius*	New Zealand, Levin	KT389503	KT389622
Didymella sancta	CBS 281.83 ^{T}	*Ailanthus altissima*	South Africa	FJ427063	KT389623
Didymella segeticola	CGMCC 3.17489 ^{T}	*Cirsium segetum*	China	KP330443	KP330414
Didymella sinensis	LC 5246	*Dendrobium officinale*	China	KY742087	KY742166
Didymella subglomerata	CBS 110.92	*Triticum* sp.	USA, North Dakota	FJ427080	KT389626
Didymella suiyangensis	CGMCC 3.18352 ^{T}	Air	China	KY742089	KY742168
***Didymella kaurna* sp. nov.**	BRIP 69579 ^{T}	*Gastrolobium celsianum*	Australia, SA, Adelaide	**MN5676780**	**MN604931**
Didymella viburnicola	CBS 523.73	*Viburnum cassioides*	The Netherlands, Wageningen	GU237879	KP330430
Neoascochyta desmazierii (outgroup)	CBS 297.69 ^{T}	*Lolium perenne*	Germany, Hohenlieth	KT389508	KT389644
Neodidymelliopsis achlydis	CBS 256.77 ^{T}	*Achlys triphylla*	Canada, British Columbia, Vancouver Island	KT389531	
Neodidymelliopsis cannabis	CBS 234.37	*Cannabis sativa*	Unknown	GU237804	KP330403
Neodidymelliopsis farokhinejadii	CBS 142850	*Citrus limon*	Iran, Dezful	KX139013	
Neodidymelliopsis longicolla	CBS 382.96 ^{T}	Soil	Israel, En Avdat, Negev desert	KT389532	
Neodidymelliopsis moricola	MFLUCC 17-1063	*Morus alba*	Russia	KY684939	KY684943
	MFLUCC 17-1064	*Morus alba*	Russia	KY684940	KY684944
Neodidymelliopsis negundinis	JZB380011	*Acer negundo*	Russia	MG564165	MG564166
	JZB380020	*Euonymus europaeus*	Russia	MH571671	
Neodidymelliopsis polemonii	CBS 109181 ^{T}	*Polemonium caeruleum*	The Netherlands	GU237746	KP330427
Neodidymelliopsis ranunculi	MFLUCC 13-0490 ^{T}	*Ranunculus* sp.	Italy	KX572338	
***Neodidymelliopsis tinkyukuku* sp. nov.**	BRIP 69592 ^{T}	*Hardenbergia violacea*	Australia, SA, Clare	**MN5676781**	**MN604932**
Neodidymelliopsis xanthina	CBS 383.68 ^{T}	*Delphinium* sp.	The Netherlands, Baarn	GU237855	KP330431
Nothophoma anigozanthi	CBS 381.91 ^{T}	*Anigozanthus maugleisii*	The Netherlands	GU237852	KT389655
Nothophoma arachidis-hypogaeae	CBS 125.93	*Arachis hypogaea*	India, Madras	GU237771	KT389656
***Nothophoma garlbiwalawarda* sp. nov.**	BRIP 69580	*Senna artemisioides*	Australia, SA, Adelaide	**MN5676782**	**MN604933**
	BRIP 69586	*Senna artemisioides*	Australia, SA, Berri	**MN5676783**	**MN604934**
	BRIP 69587	*Senna artemisioides*	Australia, SA, Berri	**MN5676784**	**MN604935**
	BRIP 69594	*Senna artemisioides*	Australia, SA, Kimba	**MN5676785**	**MN604936**
	BRIP 69595 ^{T}	*Senna artemisioides*	Australia, SA, Wudinna	**MN5676786**	**MN604937**
Nothophoma gossypiicola	CBS 377.67	*Gossypium* sp.	USA, Texas	GU237845	KT389658
Nothophoma infossa	CBS 123395 ^{T}	*Fraxinus pennsylvanica*	Argentina, Buenos Aires Province, La Plata	FJ427025	KT389659
***Nothophoma naiawu* sp. nov.**	BRIP 69583 ^{T}	*Senna artemisioides*	Australia, SA, Blanchetown	**MN5676787**	**MN604938**
Nothophoma macrospora	UTHSC DI16-199 ^{T}	*Homo sapiens*	USA, Arizona	LN880536	
***Nothophoma ngayawang* sp. nov.**	BRIP 69582 ^{T}	*Senna artemisioides*	Australia, SA, Blanchetown	**MN5676788**	**MN604939**

Nothophoma quercina	CBS 633.92	*Microsphaera alphitoides* from *Quercus* sp.	Ukraine	GU237900	KT389657
Nothophoma variabilis	UTHSC DI16-285 [T]	*Homo sapiens*	USA	LT592939	
Phoma herbarum	CBS 615.75	*Rosa multiflora* cv. Cathayensis	The Netherlands	FJ427022	KP330420
Phoma neerlandica	CBS 134.96 [T]	*Delphinium* sp.	The Netherlands	KT389535	KT389661

[1] BRIP, Queensland Plant Pathology Herbarium, Brisbane, QLD, Australia; CBS, Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands; CGMCC, China General Microbiological Culture Collection, Beijing, China; MFLUCC, Mae Fah Luang University Culture Collection, Chiang Rai, Thailand; UTHSC, Fungus Testing Laboratory at the University of Texas Health Science Center, San Antonio, Texas, USA.
[2] NSW, New South Wales; SA, South Australia; VIC, Victoria; WA, Western Australia.
[3] ITS, internal transcribed spacer region; *rpb*2, RNA polymerase II second subunit
[T] ex-type strain.

Figures

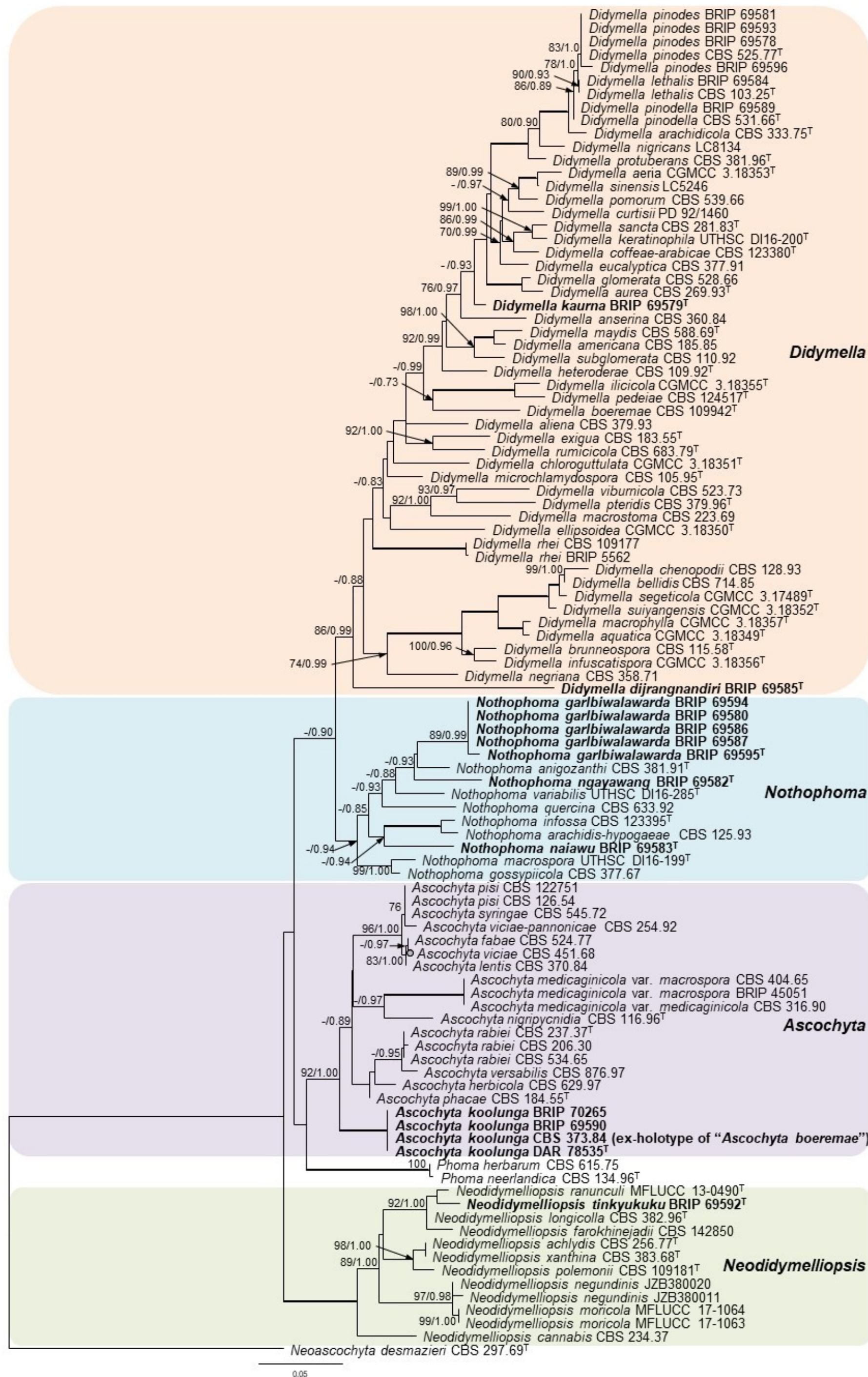

Fig. 1 Phylogenetic tree based on maximum likelihood analysis of the combined concatenated multilocus (*rpb*2, ITS) alignment. RAxML boostrap values (bs) greater than 70% and Bayesian posterior probabilities (pp) greater than 0.7 are given at the nodes (bs/pp). Novel taxa and combination introduced in this study are in bold. Ex-type isolates are marked with a superscript T (^{T}). The outgroup is *Neoascochyta desmazieri* strain CBS 297.69.

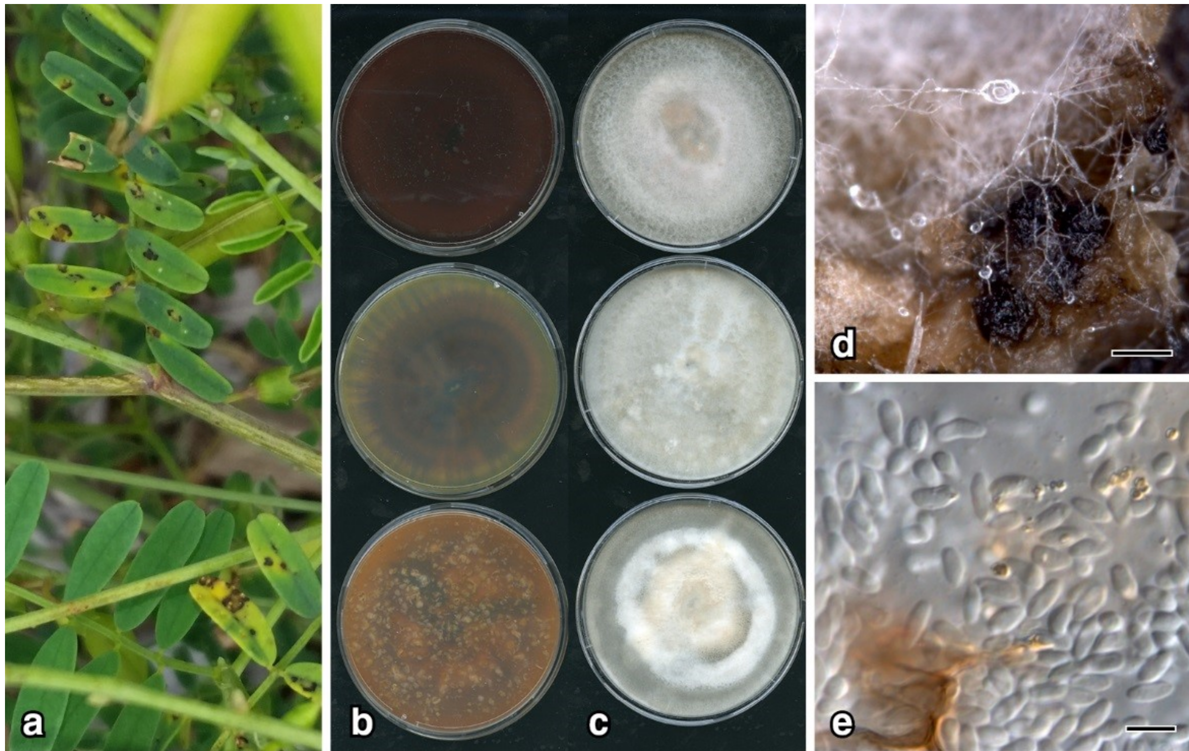

Fig. 2 *Didymella djirangnandiri* **a** Leaf lesions on *Swainsona galegifolia;* **b** 14-d old colonies, top to bottom, on PDA, MEA, OA lower surface, **c** upper surface; **d** pycnidia on CLA; **e** conidia. Scale bars: d = 200 µm; e = 7 µm.

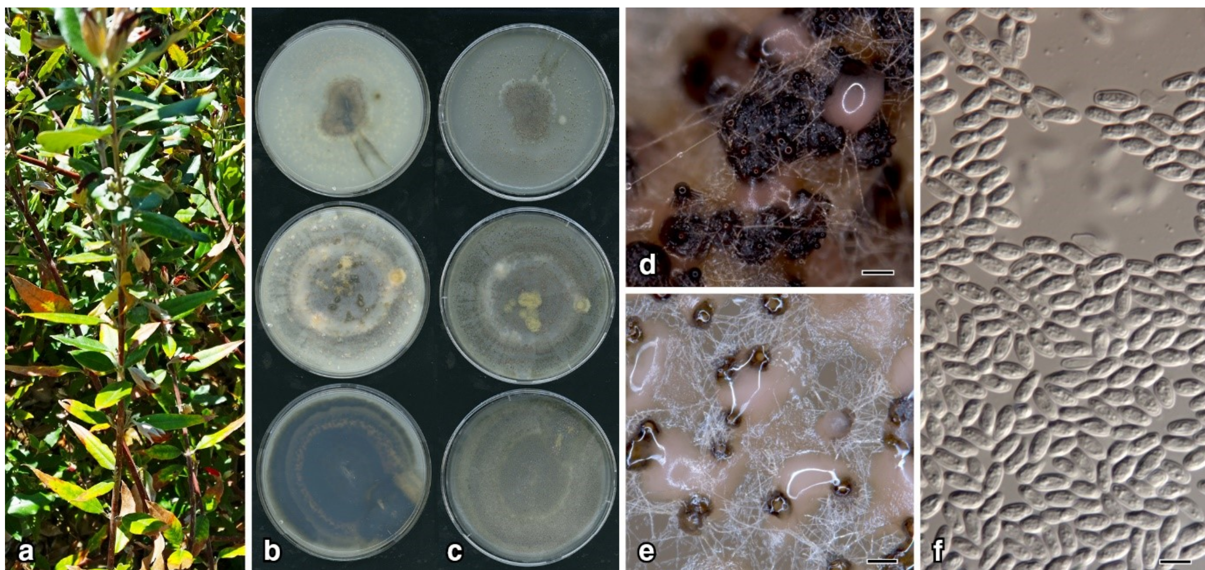

Fig. 3 *Didymella kaurna* **a** Leaf necrosis on *Gastrolobium celsianum;* **b** 14-d old colonies, top to bottom, on PDA, MEA, OA lower surface, **c** upper surface; **d** pycnidia on CLA; **e** pycnidia and pycnidial ooze; **f** conidia. Scale bars: d-e = 300 µm; f = 6 µm.

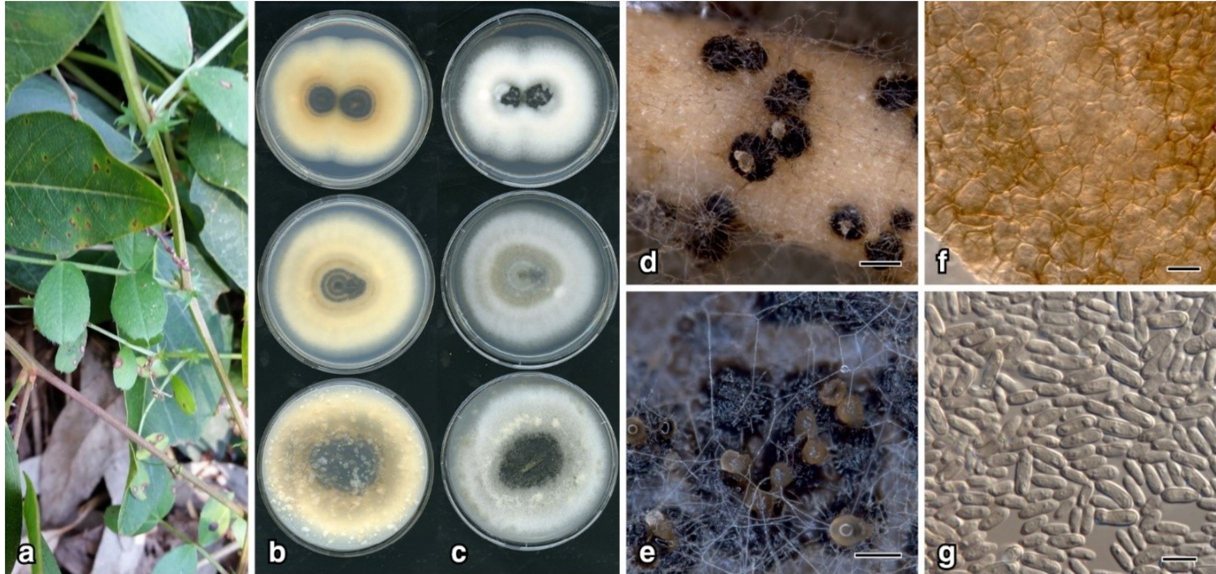

Fig. 4 *Neodidymelliopsis tinkyukuku* **a** Leaf lesions on *Hardenbergia violacea;* **b** 12-d old colonies, top to bottom, on PDA, MEA, OA lower surface, **c** upper surface; **d** pycnidia on CLA; **e** pycnidia; **f** pycnidial wall; **g** conidia. Scale bars: d-e = 300 µm; f = 10 µm; g = 7 µm.

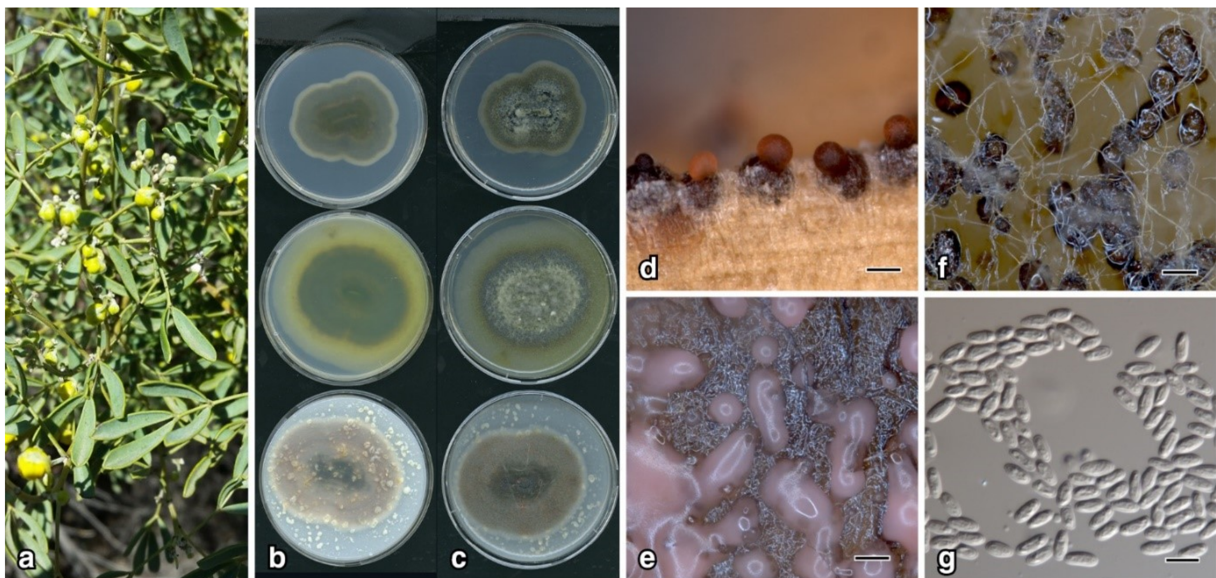

Fig. 5 *Nothophoma garlbiwalawarda* **a** Pin-prick leaf spots on *Senna artemisioides* from Wudinna SA*;* **b** 12-d old colonies, top to bottom, on PDA, MEA, OA lower surface, **c** upper surface; **d** pycnidia on CLA; **e** pycnidia and pycnidial ooze on OA; **f** pycnidia on PDA; **g** conidia. Scale bars: d-f = 300 µm; g = 7 µm.

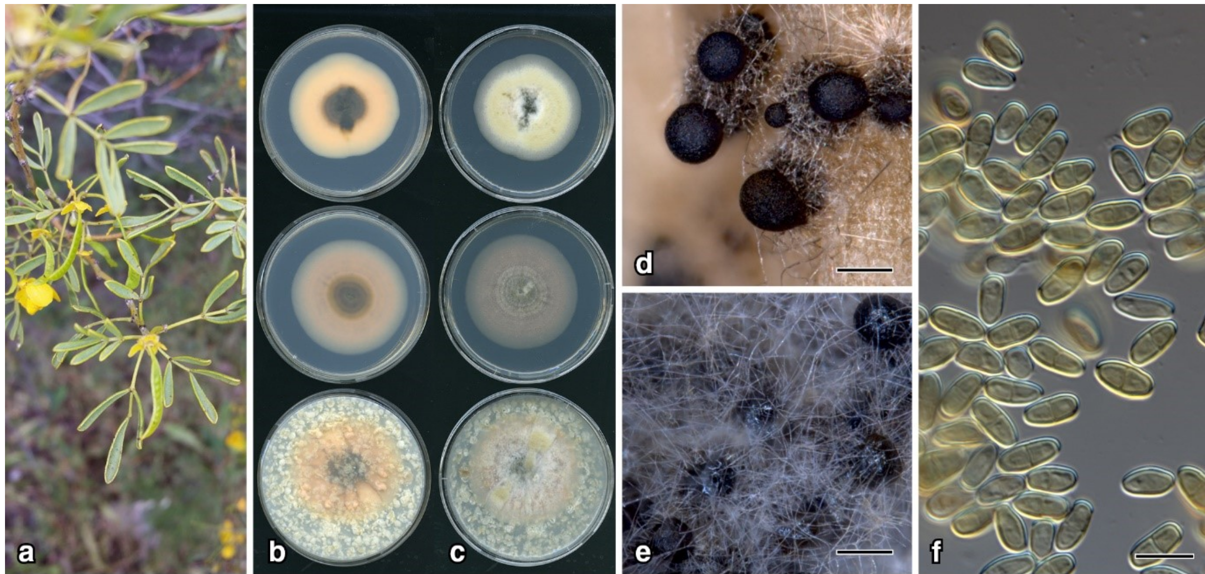

Fig. 6 *Nothophoma naiawu* **a** Pin-prick leaf spots on *Senna artemisioides;* **b** 14-d old colonies, top to bottom, on PDA, MEA, OA lower surface, **c** upper surface; **d** pycnidia on CLA; **e** pycnidia; **f** conidia. Scale bars: d-e = 300 µm; f =10 µm.

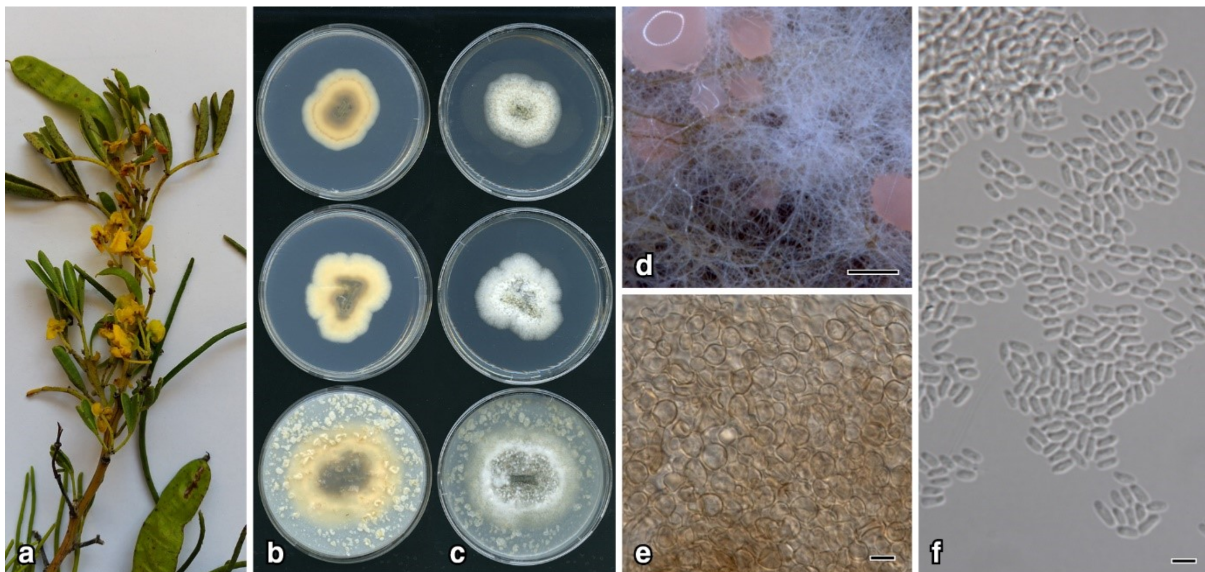

Fig. 7 *Nothophoma ngayawang* **a** Leaf and pod lesions on *Senna artemisioides;* **b** 14-d old colonies, top to bottom, on PDA, MEA, OA lower surface, **c** upper surface; **d** pycnidia; **e** pycnidial wall; **f** conidia. Scale bars: d = 250 µm; e = 8 µm; f = 3 µm

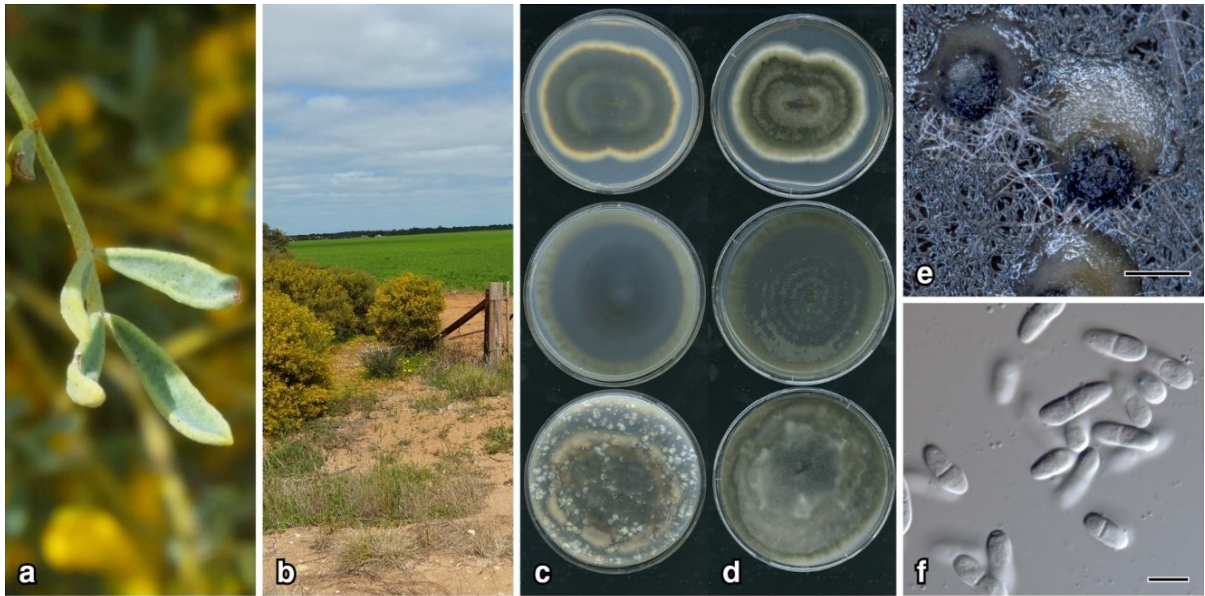

Fig. 8 *Didymella pinodes* **a** Pin-prick leaf spots on *Senna artemisioides;* **b** proximity and abundance of Senna adjacent to field pea crop in South Australia; **c** 12-d old colonies, top to bottom, on PDA, MEA, OA lower surface, **d** upper surface; **e** pycnidia and pycnidial ooze on OA; **f** conidia. Scale bars: e = 300 µm; f = 10 µm.

Fig. 9 *Didymella pinodes* leaf spots on *Vicia cracca*

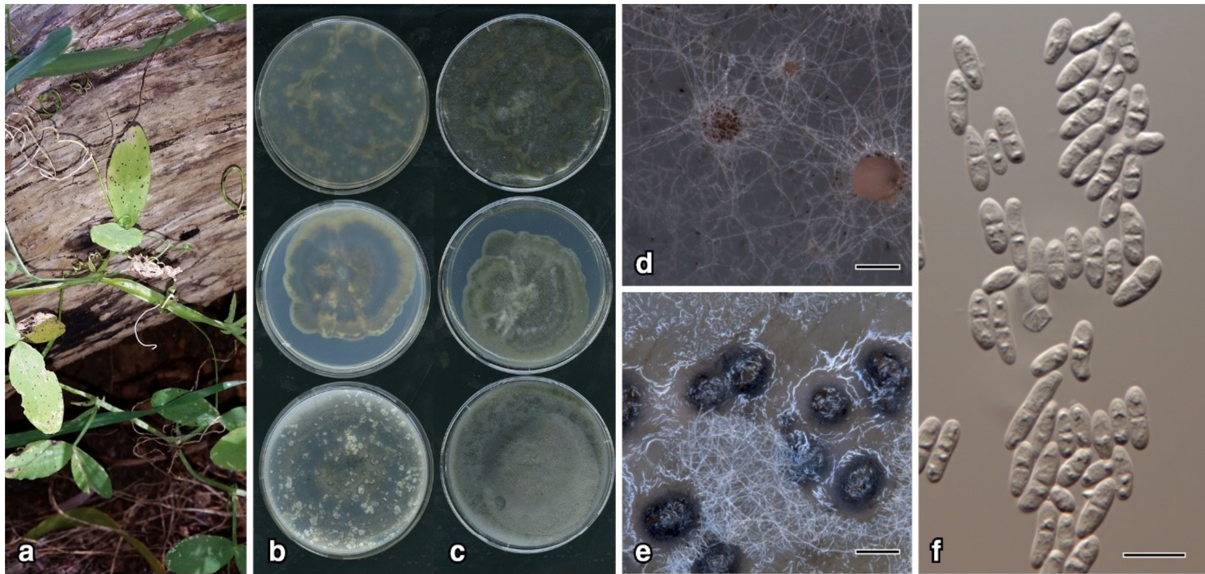

Fig. 10 *Didymella lethalis* **a** Leaf spots on *Lathyrus tingitanus;* **b** 14-d old colonies, top to bottom, on PDA, MEA, OA lower surface, **c** upper surface; **d** pycnidia on CLA; **e** pycnidia and pycnidial ooze on OA; **f** pycnidia on PDA; **g** conidia. Scale bars: d-e = 300 µm; f = 12 µm.

Chapter 4

Pathogenicity of *Nothophoma ngayawang* and *Didymella pinodes* on *Senna artemisioides* and *Pisum sativum*

Pathogenicity of *Nothophoma ngayawang* and *Didymella pinodes* on *Senna artemisioides* and *Pisum sativum*

Abstract

In order to fulfil Koch's postulates one novel species belonging to the Didymellaceae, *Nothophoma ngayawang* **sp. nov.**, isolated from *Senna artemisioides* near Blanchetown in the Murray River region of South Australia, was used to inoculate *S. artemisioides* ssp. *coriacea* and ssp. *quadrifolia* in a pathogenicity test. Pathogenicity testing was also conducted using two isolates of *Didymella pinodes*, the primary Ascochyta blight pathogen of *Pisum sativum* (field pea). One isolate of *D. pinodes* was from *S. artemisioides* collected from the Eyre Peninsula of South Australia and the second isolate, also from the Eyre Peninsula, was from *P. sativum*. *Pisum sativum* was also inoculated with all three isolates individually.

Nothophoma ngayawang caused pin-prick leaf spot symptoms on *S. artemisioides* ssp. *quadrifolia*, as seen in the field, but no leaf spot symptoms developed on ssp. *coriacea.* This fungus also caused leaf spot symptoms on *P. sativum* and these were visibly different from Ascochyta blight leaf lesions. The culture of *D. pinodes* isolated from *S. artemisioides* caused pin-prick leaf spot symptoms on *S. artemisioides* ssp. *quadrifolia* but not on ssp. *coriacea*. The pin-prick lesions were similar to the original symptoms on *S. artemisioides*. Both isolates of *D. pinodes* caused typical Ascochyta blight leaf lesions on field pea, suggesting that *S. artemisioides* could act as a reservoir of inoculum for this pathogen in field pea growing areas.

The leaf spot symptoms on *S. artemisioides* caused by *D. pinodes* and *No. ngayawang* were not easily distinguishable from each other in either field or on plants inoculated in pathogenicity tests.

Introduction

Senna artemisioides was the Australian native legume most frequently found to have leaf spots during the field collection (see Chapter 3). Three novel species of *Nothophoma* were isolated from leaves collected from *S. artemisioides*. Additionally, the primary Ascochyta blight pathogen, *D. pinodes*, was isolated from leaf spots on *S. artemisioides* from five different locations in South Australia (SA). In the host range experiments involving *P. koolunga* presented in Chapter 2, seed from *S. artemisioides* failed to germinate, resulting in this species being excluded from the range of native legumes inoculated with *P. koolunga*. Despite the difficulty experienced in germinating *Senna* species, fulfilment of Koch's postulates via pathogenicity testing was deemed necessary to investigate the veracity of these identifications.

Materials and Methods

Species identification, seed preparation, planting and growth conditions

Senna species were identified on the basis of photographic images of whole plants, leaves, flowers and seed pods sent to the State Herbarium of South Australia. One species from Blyth, mid-North, SA was deemed, using the taxonomic key of Randell (1989), "fairly unequivocally" to be the hybridogenic *Senna artemisioides* ssp. *coriacea* (P. Lang, State Herbarium of South Australia, 2018, *pers. comm.*). The second, from Mt Wudinna, Eyre Peninsula, SA was placed closest to *Senna artemisioides* ssp. *quadrifolia,* but some hybridization with ssp. *petiolaris* may have been involved based on the observed flattening of the petioles.

Seed of *S. artemisioides* ssp. *coriacea* and ssp. *quadrifolia*, was obtained from the SARDI Australian Pastures Genebank, Adelaide, SA and scarified before sowing as described in Chapter 2. Planting soil, pot size and controlled growth conditions in CER were also as described in Chapter 2. After planting, the germination period was extended to allow for slow germination of some seed. The eventual time to inoculation was 5 months from planting. From

the 95 seeds of *S. artemisioides*, ssp. *coriacea*, sown at four seeds per pot, only 20 plants grew in a total of 14 pots, with either one of two plants per pot. From the same number of seeds of *S. artemisioides* ssp. *quadrifolia*, sown at the same rate per pot, only 13 plants grew in a total of 11 pots. Ten pots of field pea cv. Kaspa, were planted 19 days prior to the inoculation date at four seeds per pot, as described in Chapter 2.

One pot with one plant of each *Senna* subspecies and one pot with four plants of *P. sativum* served as controls. Due to the poor germination of both *Senna* subspecies, and use of one plant as a control, there remained only 12 plants of ssp. *quadrifolia* available for inoculation with the three isolates, resulting in four plants for each isolate in a total of 10 pots. For ssp. *coriacea* there remained 19 plants available for inoculation with the three isolates, giving six plants for each isolate, with one extra plant inoculated with *No. ngayawang*, in a total of 13 pots. Table 1 summarises the allocation of pots and plants for inoculation with each isolate.

Table 1: Number of pots and plants allocated to be inoculated with individual isolates (number of plants per plant/isolate combination)

Plant Species	***No. ngayawang***	***D. pinodes* from *S. artemisioides***	***D. pinodes* from *P. sativum***	**Control**
S. artemisioides ssp. *quadrifolia*	4 plants in 4 pots (4 single plants)	4 plants in 3 pots (4 single plants)	4 plants in 3 pots (4 single plants)	1 plant/1 pot
S. artemisioides ssp. *coriacea*	7 plants in 4 pots (7 single plants)	6 plants in 4 pots (6 single plants)	6 plants in 4 pots (6 single plants)	1 plant/1 pot
P. sativum cv. Kaspa	12 plants in 3 pots/4 per pot (12 single plants)	12 plants in 3 pots/4 per pot (12 single plants)	12 plants in 3 pots/4 per pot (12 single plants)	4 plants in 1 pot/4 per pot (4 single plants)

Fungal isolates

It was initially planned to inoculate both *S. artemisioides* subspecies individually with each of the three novel *Nothophoma* species, and also to compare disease incidence and severity caused by two *D. pinodes* isolates from *S. artemisioides*. *Phoma koolunga* was also to be tested on the *Senna* subspecies because neither was included in the host range experiments described in Chapter 2, but also to compare symptoms with the *D. pinodes* isolates on *Senna*. However, insufficient germination of both *Senna* subspecies meant that only one *No. ngayawang* and one *D. pinodes* isolate from *S. artemisioides* could be chosen. For comparison, one isolate of *D. pinodes* from *P. sativum* collected in the field was included instead of *P. koolunga*.

The single conidium-derived isolates of *D. pinodes* and *No. ngayawang* used were; *D. pinodes* isolate 1, BRIP 96596 (collected in 2017 from *S. artemisioides* at the Mt Wudinna walking track car park, SA), isolate 2 (collected in 2017 from a commercial crop of *P. sativum* at Wudinna, SA), and *No. ngayawang*, BRIP 69582 (collected in 2016 from *S. artemisioides* on a highway roadside, Blanchetown, SA). Mycelial plugs of each isolate, retrieved from storage at 4 ºC, were placed onto potato dextrose agar (PDA, Oxoid®) plates. Eight plates were prepared for each isolate and incubated under 12 h black and fluorescent light/12 h darkness cycle at 22 ºC. After 16 days of incubation, conidial suspensions were prepared and adjusted to between 1.0 and 1.5 × 10^5 conidia/mL. Tween 20 (0.01%) was added as a surfactant (Davidson et al. 2012).

Plant inoculation

At the time of inoculation, plants were removed from the tent within the CER to separate areas for inoculation to avoid cross-contamination of isolates. Control pots were kept separate. One control pot for each plant species was mock-inoculated with sterile reverse osmosis water supplemented with 0.01 % Tween 20 from a hand-held sprayer. Suspensions of conidia of each

isolate were individually sprayed on plants at approximately 2 mL of suspension per plant. The small volume of inoculum obtained at the required concentration (1.0 - 1.5 × 10^5 conidia/mL) did not enable spray until run off for any of the isolates.

After inoculation, the 10 or 11 pots for each isolate were returned to tents within the CER in separate trays, with pots arranged in a completely randomised block design in the conditions already described in Chapter 2. For the first 8 h post-inoculation a humidifier (ionmax™ Andatech Pty Ltd) was used in each tent at medium level II mist setting to provide humid conditions conducive for infection and disease development. In this experiment the humidifier was set to run continuously for 48 hours as in the host range experiments described in Chapter 2. For unknown reasons the humidifier only ran for 8 h post-inoculation therefore it was used again for four additional 8 h periods within the first 96 h post-inoculation.

Disease assessment and re-isolation

Plant response to inoculation was visually assessed at days 7, 9, 11 and 35 days post-inoculation (dpi). The ability of each fungal species to cause leaf spot symptoms was rated as "yes, or no", when compared to control plants, with the estimated percentage of leaf area with disease symptoms recorded at 11 d only, for 12 to 13 randomly chosen inoculated leaves for each species. The average percentage was then calculated. If leaf area diseased was too small to quantify, it was recorded as "trace". A statistical analysis was not performed due to insufficient numbers of replicates and inability to quantify percentage leaf area diseased. Photographs were taken of whole plants and leaves, with and without symptoms, of each species x isolate combination on assessment days 7, 9, 11 and 35 dpi using a Samsung Galaxy S8 mobile phone camera.

To satisfy Koch's postulates, leaf samples from inoculated plants showing leaf spot symptoms were collected at 11 dpi. Leaves were surface disinfected following the method of

the Royal Botanic Gardens Sydney (M. Laurence, Botanic Gardens and Centennial Parklands 2017, *pers. comm.*) as described in Chapter 2. Leaf segments were then placed on APDA medium and incubated as described in Chapter 2. After 12 days the resulting fungal colonies were identified using macroscopic and microscopic morphological characteristics, including cultural growth, colour, patterns of zonation and conidial size. Comparisons were made with published descriptions of *D. pinodes* and the description of *No. ngayawang* provided in Chapter 3.

Results

A summary of the results for the percentage leaf area diseased for each isolate x species combination and the re-isolation of the fungus from leaf lesions is provided in Table 2. Figure 1a shows the original specimen from which *No. Ngayawang* was isolated. A culture of *No. ngayawang* used for inoculum is shown in Fig. 1b. Small pin-prick lesions were observed on *S. artemisioides* ssp. *quadrifolia*, which were just visible to the naked eye at 11 dpi (Fig. 1c), becoming more pronounced by day 35 (Fig. 1d). The average leaf area diseased ranged from trace to 2% at 11 dpi. *No. ngayawang* could not be re-isolated from the leaf lesions. *S. artemisioides* ssp. *coriacea* showed no leaf lesions. *No. ngayawang* caused leaf lesions on *P. sativum*. The average leaf area diseased was 7.8% 11 dpi (Fig. 1e) and did not increase substantially after day 11. *No. ngayawang* was re-isolated from the leaf lesions on day 11 (Fig. 1f.) There were no leaf spots on leaves of the control plants.

Figure 2a shows the original specimen of *P. sativum* from which *D. pinodes* was isolated. A culture of *D. pinodes* used for inoculum is shown in Fig. 2b. Fig. 2c shows a culture of *D. pinodes* re-isolated from *P. sativum.* Typical Ascochyta blight leaf symptoms were clearly visible at day 9 (Fig. 2d) with further leaf necrosis observed at day 35 and some new healthy-looking shoots emerged from the base of infected plants (Fig. 2e). The average leaf area

diseased was 10.1% at 11 dpi. *Didymella pinodes* was re-isolated from leaf lesions at 11 dpi. All control plants were without leaf lesions. This isolate of *D. pinodes* did not cause any leaf lesions on either *Senna* species (not shown).

Figure 3a shows the original specimen of *S. artemisioides* from which *D. pinodes* was isolated. A culture of *D. pinodes* used for inoculum is shown in Fig. 3b. Fig. 3c shows a culture of *D. pinodes* re-isolated from *P. sativum.* Small pin-prick lesions were observed on *S. artemisioides* ssp. *quadrifolia*, which were visible to the naked eye at 7, 9 and 11 dpi (Figs 1d, e and f respectively). At day 35 dpi (Fig. 1g) the lesions remained small and did not appear to expand or coalesce but did become more pronounced and greater in number. The average leaf area diseased was 12.3% at 11 dpi. *S. artemisioides* ssp. *coriacea* showed no leaf lesions. *D. pinodes* could not be re-isolated from the leaf lesions on *S. artemisioides*. On *P. sativum,* the isolate caused symptoms typical of Ascochyta blight of field pea, clearly visible by day 9 (Fig, 3h) with necrosis continuing to develop to d 35 (Fig. 3i). The average leaf area diseased was 9.6 % at 11 dpi. *D. pinodes* was re-isolated from inoculated leaves with lesions at 9 dpi. All control plants were without leaf spots.

Table 2: Summary of species x isolate leaf area disease at 11 dpi and results of re-isolation

Plant Species	***No. ngayawang* from *S. artemisiodes* (Fig. 1)**	***D. pinodes* from *P. sativum* (Fig. 2)**	***D. pinodes* from *S. artemisioides* (Fig. 3)**
S. artemisioides ssp. *quadrifolia*	Average leaf area diseased – trace to 2% Re-isolation - No	Average leaf area diseased – 0% Re-isolation - NA	Average leaf area diseased – 12.3% Re-isolation - No
S. artemisioides ssp. *coriaceae*	Average leaf area diseased – 0% Re-isolation - NA	Average leaf area diseased – 0% Re-isolation - NA	Average leaf area diseased – 0% Re-isolation - NA
P. sativum cv. Kaspa	Average leaf area diseased – 7.8% Re-isolation - Yes	Average leaf area diseased – 10.1% Re-isolation - Yes	Average leaf area diseased – 9.6% Re-isolation - Yes

Discussion

Inoculation of *S. artemisioides* ssp. *quadrifolia* with *No. ngayawang*, in controlled conditions, resulted in pin-prick leaf lesions similar to those observed on *S. artemisioides* in the field and from which *No. ngayawang* was originally isolated. *No. ngayawang* was also able to cause leaf lesions on *P. sativum* and could be re-isolated from *P. sativum.* Similarly, *D. pinodes* isolated from naturally infected *S. artemisioides* was able to infect *S. artemisioides* ssp. *quadrifolia* and *P. sativum* in controlled conditions and was able to be re-isolated from *P. sativum*. However, *D. pinodes* isolated from *P. sativum* caused lesions on leaves of *P. sativum*, from which it was re-isolated, but did not cause symptoms on either subspecies of *S. artemisioides*. These results regarding infection and re-isolation of these two fungal isolates on *P. sativum* and *S. artemisioides* require additional investigation. Confirmation of Koch's postulates was demonstrated with respect to *D. pinodes* from *P. sativum*, but not fulfilled with respect to *S. artemisioides* for *No. ngayawang* and *D. pinodes* as re-isolation was not achieved. As germination of *Senna* plants from seed can be unreliable, established tube stock could be used to provide a suitable number of replicates for a statistical analysis and confirmation of Koch's postulates could be attempted. In any new experiments, *D. pinodes* isolates from field pea should again be included as well as *P. koolunga.*

The results for the testing of pathogenicity were confounded by several sub-optimal experimental conditions. Cultures produced fewer conidia that expected, and some plates were contaminated with bacteria leading to the lower than expected volume of inoculum to obtain an inoculum concentration of between 1.0 and 1.5 x 10^5 conidia/mL. Inoculum concentrations used in similar experiments have ranged from 1.0 x 10^5 to 1.0 x 10^6 spores per ml (Davidson et al. 2009a, Fondevilla et al. 2005, Le May et al. 2014, Onfroy et al. 1999). The concentration used in the host range experiment detailed in Chapter 2 was 1.5 x 10^5 and this was the concentration aimed for when pathogenicity testing. The small volumes meant not being able to spray to run

off, resulting in uneven distribution of inoculum, which contributed to variable areas of leaf symptom development among plants in the same pot. Additionally, continuous high humidity for the first 48 hours post-inoculation was not able to be maintained for unknown reasons as the misting equipment was the same brand, but not the same unit, as used in the host range experiments detailed in Chapter 2. A period of at least 24 hours of high humidity has been documented as an important environmental parameter when evaluating the ability of *D. pinodes* isolates to cause leaf disease symptoms (Fondevilla et al. 2005, Jha et al. 2012, Le May et al. 2009, Skoglund et al. 2011, Tran et al. 2015b). The environmental conditions conducive for *No. ngayawang* to cause disease are unknown. The temperature within the CER, despite choosing temperature settings to match the host range experiments in Chapter 2, was lower than expected. Instead of being 22 to 23 °C during the 12-hour day period, the Tiny Tag® sensor recorded a temperature between 15.5 and 17.8 °C for up to 33 days after inoculation. This was noticed only at the end of the 35-day period when the temperature recording was downloaded from the sensor and could be attributed to gaps in the plastic tent door where the ageing Velcro seal allowed cool air to enter. As a consequence of these conditions, the disease development may have been impaired.

Another experimental variable that could have influenced the results concerns the identification of the *S. artemisioides* subspecies observed in the field and those used in the pathogenicity testing. Randell (1989) comments that: *Senna* species "readily form highly fertile autotetraploids", meaning hybrid individuals. The resultant complexity of the genetics and morphology of this genus makes accurately naming species difficult and rather arbitrary. It is therefore possible that the *Senna* subspecies from which fungal isolates were obtained in the field might not have been *quadrifolia* nor the hybridogenic subspecies *coriacea*, but rather more distant relatives or hybrids. If the subspecies used were not the original hosts of the isolates

identified, and tested for confirmation of Koch's postulates, the validity of the pathogenicity test could be questioned.

The leaf lesions observed on *S. artemisioides* ssp. *quadrifolia* inoculated with either *No. ngayawang* or *D. pinodes* were small and restricted and were very similar to those observed in the field; this could be considered more indicative of a hypersensitive response than disease. *Didymella pinodes* and *No. ngayawang* were not re-isolated from *S. artemisioides*, only from infected *P. sativum* leaf tissue, which suggests that *S. artemisioides* may have activated an autoimmune-like hypersensitive defence mechanism which confounded re-isolation in the experimental conditions. A hypersensitive response is directed plant cell death at the site of infection, limiting pathogen access to water and nutrients. The death of a small number of cells is designed to save the rest of the plant. Such a hypersensitive response is not generalised, more likely pathogen-specific, triggered when disease-causing effector molecules introduced into the host plant cells by the pathogen are detected (Chakraborty et al. 2018, Deverall 1977, Freeman and Beattie 2008, Zhang et al. 2004). Nothing is known about effectors in this system.

The results for the *D. pinodes* isolate from *S. artemisioides* show that it can infect *P. sativum* and, assuming conditions conducive to spread into a field pea crop occurred, *Senna* species could be a source of inoculum for this Ascochyta blight pathogen. *Senna artemisioides* subspecies are found throughout Australia, and are indigenous to 10 botanical regions in SA, which include the field pea growing regions of Eyre Peninsula, Northern Lofty, Murray and Yorke Peninsula (http://plantselector.botanicgardens.sa.gov.au/). However, without further investigation, the role of this host species as another inoculum source for Ascochyta blight is uncertain. The likelihood that *D. pinodes* might spread via rain splash of conidia from *S. artemisioides* to field pea plants would appear low, given the small size of leaf lesions and lack of any visible conidia on the leaf surface. Examination of *Senna* in the field to look for evidence of ascospore-producing structures on leaf litter at soil level could provide additional

information to be able to discount the possibility of air-borne ascosporic transmission of disease, or release into soil. Soil at the base of *Senna* plants could be tested to ascertain if inoculum of *D. pinodes* is present. If *Senna* does prove to be a host of viable and transmissible spores, growers might have to consider removal of plants close to field pea cropping paddocks. *Senna* is not an endangered, or protected native legume, so removal is possible. That *No. ngayawang* could also cause leaf spots on *P. sativum* would suggest that, given proximity to a field pea crop and favourable environmental conditions, this species could contribute to leaf damage within a field pea crop. However, it is less likely that it would be a significant pathogen, given the prevalence and severity of symptoms caused by Ascochyta blight pathogens. Supportive of this is that although at day 11, the severity of leaf disease on field pea was similar for *No. ngayawang* and *D. pinodes*, unlike *D. pinodes,* the percentage leaf area diseased and severity of leaf symptoms caused by *No. ngayawang* did not increase substantially after day 11.

New experiments to test the pathogenicity of all three novel *Nothophoma* species isolated from *S. artemisioides* could provide information on epidemiology of these fungi. For the *D. pinodes* isolates from *S. artemisioides*, pathogenicity testing should be repeated using both *S. artemisioides* and *P. sativum*, in conditions conducive for disease development, to assess the potential of *S. artemisioides* to act as an alternative inoculum source and, if it can, what consequences this might have for the spread of Ascochyta blight of field pea.

Acknowledgements

Thanks to Dr Steve Hughes (South Australian Research and Development Institute) for sourcing native *Senna* seed, and Dr Peter Lang (State Herbarium of South Australia) for the identification of *Senna artemisioides* subspecies.

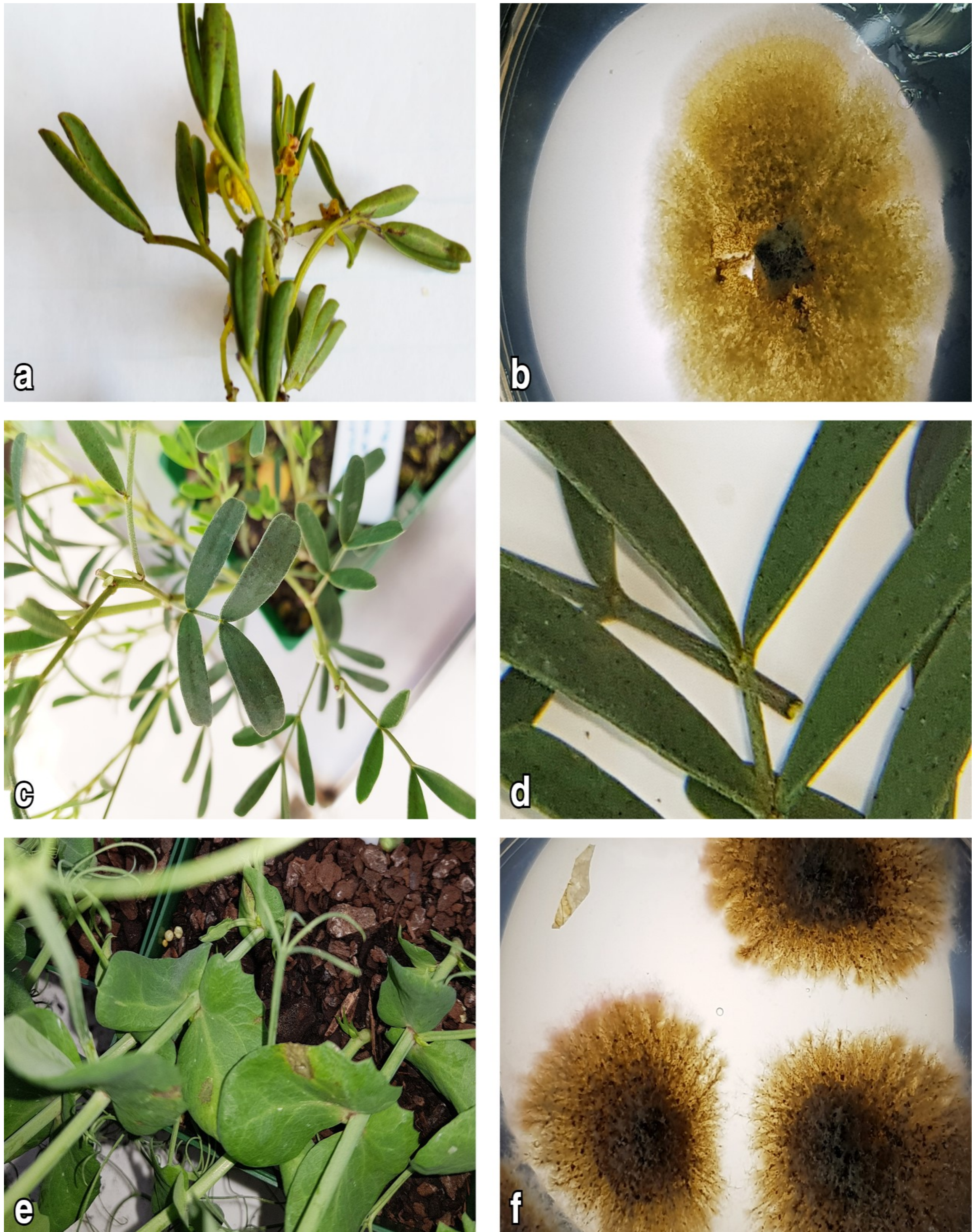

Fig. 1a Leaf lesions on *S. artemisioides*, **b** 16 d-old culture on potato dextrose agar of *Nothophoma ngayawang* used for inoculum, *S. artemisioides* leaf lesions days post-inoculation **c** 11 dpi, **d** 35 dpi, **e** *Pisum sativum* leaf lesions 11dpi with *No. ngayawang*, **f** 12 d-old culture of *No. ngayawang* on acidified potato dextrose agar isolated from *P. sativum* leaf lesions shown in **e** .

Fig. 2 **a** Ascochyta blight on *Pisum sativum* from which *Didymella pinodes* was isolated, **b** 16 d-old culture of *D. pinodes* on potato dextrose agar used for inoculum, **c** 12 d-old culture on acidified potato dextrose agar of *D. pinodes* isolated from *P. sativum* leaf lesions shown in **d**, *P. sativum* leaf lesions days post-inoculation **d** 9 dpi, **e** 35 dpi.

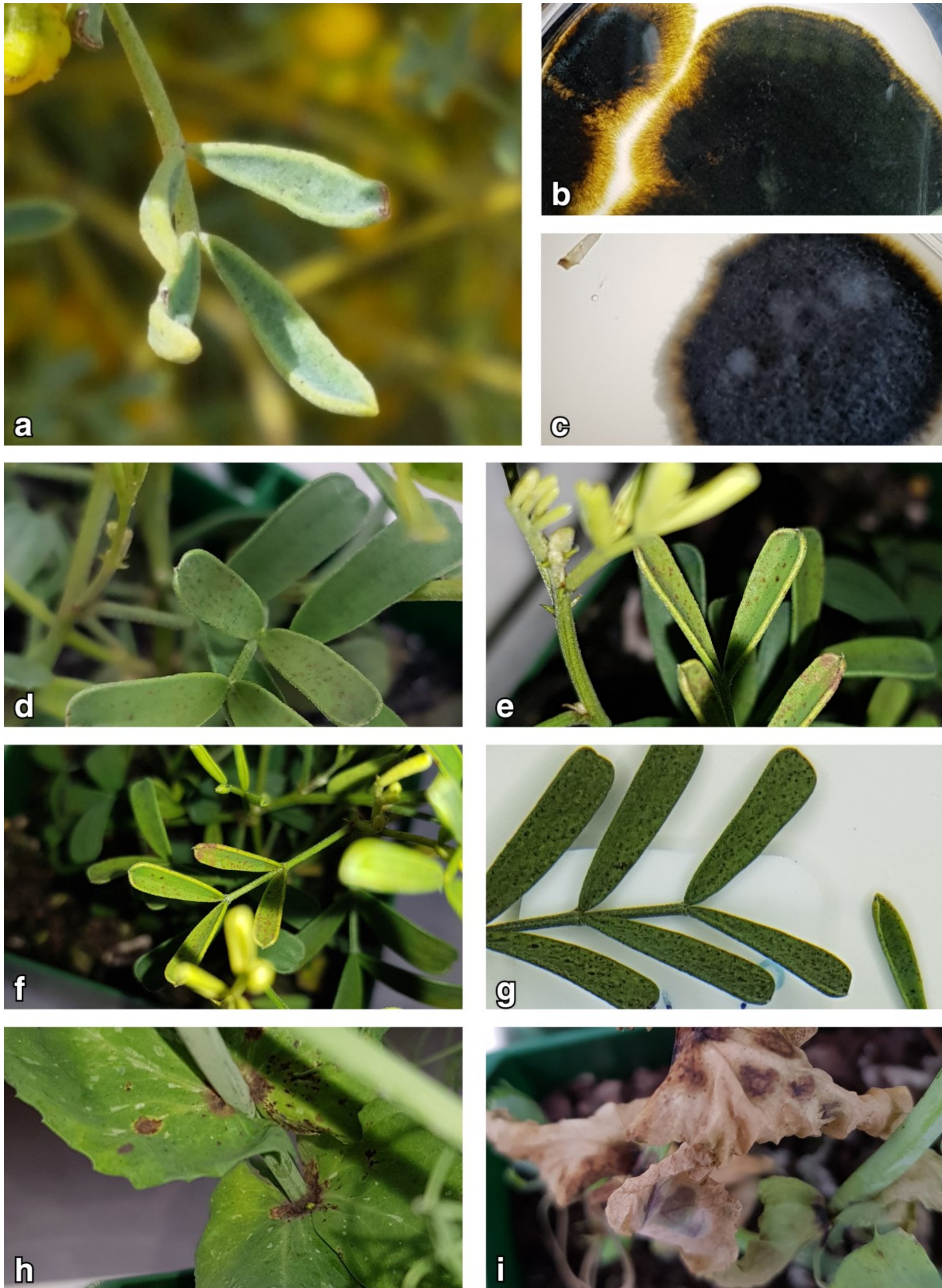

Fig. 3 **a** *Senna artemisioides* field specimen with pin-prick leaf lesions, **b** 16 d-old culture on potato dextrose agar of *Didymella pinodes* from *S. artemisioides* used for inoculum, **c** 12 d-old culture on acidified potato dextrose agar of *D. pinodes* isolated from *Pisum sativum* leaf lesions shown in **h**, *S. artemisioides* leaf lesions at days post-inoculation **d** 7 dpi, **e** 9 dpi, **f** 11 dpi, **g** 35 dpi, *P. sativum* leaf lesions days post-inoculation, **h** 9 dpi, **i** 35 dpi.

Chapter 5

General discussion

General discussion

This research was initiated to address one overall question; could *P. koolunga* have origins in Australian native, pasture or naturalised legumes? Inoculation with *P. koolunga* resulted in disease of a broad range of legumes in controlled environmental conditions. Of the 41 legumes tested all but *C. arietinum* (chickpea) showed some degree of susceptibility. However, *P. koolunga* was not isolated from diseased leaves of Australian native legumes collected from any areas sampled. Instead, the Ascochyta blight pathogen *D. pinodes* was isolated from native *S. artemisioides* and six new species of Didymellaceae were found. The data in this study suggesting a broad host range of *A. koolunga* together with the failure to isolate the fungus from lesions occurring naturally on native legumes do not, at this time, support an indigenous origin for *A. koolunga*. A more in-depth collection and intensive morphological and molecular study is needed to substantiate or refute this suggestion.

Disease development in the host range experiments occurred within 2–4 days of inoculation and infection in the most susceptible species continued to progress for the 12 dpi period. While *P. koolunga* did not cause leaf disease on the one *C. arietinum* cultivar used, *C. arietinum* has been reported to be susceptible to the Ascochyta blight pathogen, *D. pinodes*, with variability in incidence noted among isolates and cultivars tested but always with low disease severity. All accessions were resistant to one isolate (Barilli et al. 2016). An expanded number of chickpea cultivars, including those susceptible to *D. pinodes,* could be inoculated with a number of isolates of *A. koolunga* individual, disease incidence and severity compared. This would ascertain if other chickpea cultivars are susceptible to *A. koolunga* and if there might be a risk for cross-infection between chickpea and field pea crops. In the future, it would also be appropriate to expand the research on investigating the potential for vetch, lentil or other crops grown in rotation with field pea to serve as alternative hosts of *A. koolunga*, and to serve as a source of inoculum for field pea crops.

The extent of susceptibility to *A. koolunga* of wild type *Pisum* species was in contrast to reports of some degree of resistance to the primary Ascochyta blight pathogen, *D. pinodes* (Barilli et al. 2016, Fondevilla et al. 2005). A comparison of the evolutionary development of the genomes within the genus *Pisum*, including geographic origins and migrations, might provide some insight into the evolution of disease susceptibility of *Pisum* species to the Ascochyta blight pathogens. In a recent phylogenetic analysis of sequence data for *Pisum*, Kreplak et al. (2019) reported that *P. fulvum* clustered separately from *P. sativum*. *Pisum sativum* accessions clustered according to cultivated status (wild or cultivated), geographical origin and usage type (fodder, dry or fresh seeds), with the common ancestor likely to be *P. elatius*. Germination of freshly harvested seed after 7 days is a key trait of domestication and experiments showed that wild type *P. fulvum* and *P. elatius* did not germinate within 7 days when compared to cultivated *P. sativum* accessions (Kreplak et al. 2019). Comparison of genome sequence differences among *Pisum* species with the genome sequence data of each Ascochyta blight pathogen could enable the production of an evolutionary timeline for both plant and pathogen to ascertain if co-evolutionary events have occurred, thereby explaining the possible origins of each Ascochyta blight pathogen relative to each *Pisum* species.

Following the host range study, the intention of the field collections was to collect samples from within and around field pea crops, including the neighbouring natural environmental spaces of national parks and conservation areas, to investigate if *P. koolunga* could be found in association with leaf disease on legumes other than field pea. Looking for species that were shown to be susceptible in the host range experiments was of particular interest. Visual assessment of Ascochyta blight within field pea crops visited in 2017 revealed the overall incidence of disease to be low to moderate and variable within and among states (NSW, SA and VIC). As only one isolate of *P. koolunga* was obtained from the field pea samples collected, the general incidence of this pathogen in 2017, compared to the more frequently isolated *D.*

pinodes and *D. pinodella*, was regarded as low. The incidence of *P. koolunga* associated with Ascochyta blight of infected field pea is known to fluctuate from one season to the next for reasons as yet unknown (J.A. Davidson, 2018, *pers. comm.*). In a season when *P. koolunga* is more frequently isolated from field pea it might be found more readily in nearby native or pasture legumes. To confirm presence on legumes other than field pea, a systematic longitudinal survey of native and pasture legumes over several seasons could be directed to areas where the DNA-based soil test, PREDICTA® B, which is used to detect the presence of *P. koolunga*, indicates high levels of inoculum (Davidson et al. 2011). "PREDICTA® B (B = broadacre) is a DNA-based soil testing service that helps grain and pulse producers identify which soil-borne pathogens pose a significant risk to their crops before seeding so steps can be taken to minimise risk of yield loss" (https://pir.sa.gov.au/research/services/molecular_diagnostics/predicta_b). Additional high risk areas to target for surveys could be found by consulting the forecasting model for Ascochyta blight, Blackspot Manager, around the time of planting field pea crops (Salam et al. 2011b). To complement surveys in areas likely to have high inoculum incidence, a focused survey specifically targeted to find the native legume species identified in the host range study as susceptible could provide additional information on fungal biodiversity and identify if *P. koolunga* was present.

Phoma koolunga was identified and named in 2009 in SA as a new species within the Ascochyta complex. However, it appears to have been present in field pea, causing Ascochyta blight disease, for over 30 years. The prior taxonomic classification as *M. phaseolina* by Ali and Dennis (1992) was changed to *P. koolunga* because of the advent of molecular techniques that enabled SBI and SBC. Now, our phylogenetic investigations of novel Didymellaceae species revealed strong phylogenetic evidence that *P. koolunga* should be re-named because there is a species synonymy with *A. boeremae*, as presented in Chapter 3, with the proposed change in nomenclature to *Ascochyta koolunga*. To confirm the change in nomenclature, PCR

amplicons of two isolates of *P. koolunga* were sequenced at two additional loci, LSU and *tub*2, by colleague Dr Yu Pei Tan, following Chen et al. (2017), and produced sequences identical to *A. boeremae* (results not included). The taxonomic descriptions of *P. koolunga* in Davidson et al. (2009a) and *A. boeremae* in Chen et al. (2017) differ so a comparison of morphology of cultures established on the same media and incubated in the same environmental conditions at the same time would be beneficial to standardise the taxonomic description. A comprehensive description also requires the testing of pathogenicity of both isolates on field pea to compare the phenotypes of disease symptom development on field pea.

From the field collections no isolates of interest came from national parks or conservation areas. Most of the legumes observed in natural ecosystems were healthy and lacked leaf spots. All samples of interest came from legumes in proximity to field pea crops, within crops, or on neighbouring verges and roadsides, except for the two isolates from botanic gardens. *Didymella pinodes* was isolated from the native legume *S. artemisioides* from numerous locations across SA. It is not known if this native plant is acting as an alternative host for this Ascochyta blight pathogen, nor if it could also serve as a host for *P. koolunga*. However, by testing the pathogenicity of one isolate obtained from *S. artemisioides* it was confirmed that this isolate could infect both *S. artemisioides* ssp. *quadrafolia* and *P. sativum*. As discussed in Chapter 4, re-isolation from ssp. *quadrifolia* was not successful and the pathogenicity experiment was sub-optimal due to several experimental problems. However, the question regarding *Senna* as a host of *D. pinodes* with potential to harbour inoculum that could be spread to field pea crops remains. Further research on *S. artemisioides* to determine its status as a host for *D. pinodes* is required, given the small and restricted leaf spot symptoms exhibited.

In summary, *P. koolunga* has a broad host range amongst legumes in controlled environmental conditions, favourable for disease development, which includes Australian native legumes. Further research has been suggested above to answer two new questions raised

as a result of the host range study, which are: why does *A. koolunga* not cause leaf spot disease on the chickpea cultivar used in this study, a result which is consistent with the earlier report by Ali and Dennis (1992); and why were wild *Pisum* species just as susceptible to *A. koolunga* as the cultivated *P. sativum* cv. Kaspa whereas other researchers have suggested that wild type *Pisum* species show some degree of resistance (Fondevilla et al. 2005, Jha et al. 2012).

Phylogenetic analyses conducted on field samples identified six novel fungi isolated from native legumes and identified that the re-naming of *P. koolunga* as *Ascochyta* would be appropriate to correct the nomenclature. The significance of the phylogenetic analyses that revealed the occurrence of *D. pinodes* on *S. artemisioides* requires further investigation to ascertain its status as a potential alternative host, the extent of pathogenicity and implications for epidemiology of Ascochyta blight on field pea.

Systematic surveys of potential legume hosts of *A. koolunga*, other than field pea, have been proposed using existing monitoring technology to optimise identification of *A. koolunga* in locations and years with high inoculum levels. Such surveys would also contribute to a biodiversity inventory of leaf spot fungi on Australian native legumes to identify any further novel species. However, even in a year of abundant infection by *A. koolunga*, if it is possible to isolate *A. koolunga* from Australian native species, would this support a claim to indigenous origins? Possibly yes, but it is also important for researchers in other field pea growing countries around the world to actively look for *A. koolunga* locally. The current PREDICTA® B test developed at SARDI for use in Australia is based on a DNA sequence from the ITS region in the Ascochyta blight pathogens and could be shared with other researchers. There is one test for *A. koolunga* and another test that detects *D. pinodes* and *D. pinodella*, as these latter two species are identical in the ITS region (J.A. Davidson, 2020 *pers. comm*). Further DNA based tests using *rpb*2 sequences of the pathogens might be able to discriminate all three species. If *A. koolunga* was not able to be isolated from field pea crops in other parts of the world this

would help strengthen a claim to an Australian origin. If Australian native, pasture or naturalised legumes, in future research, reveal the presence of *A. koolunga*, then this would be new knowledge to bring to the attention of the Australian field pea industry. Independent and government agricultural, agronomy extension and research organisations will need to provide guidance on how to manage these potential alternative legume hosts in field pea growing regions of Australia.

Appendix 1

Passaging and preparation of *P. koolunga* isolates for host range studies

The use of moderately aggressive isolates of *D. pinodes* in host range and resistance studies involving wild type accessions is recommended to facilitate the identification of subtle differences in responses to infection (Fondevilla et al. 2007, Jha et al. 2012).

Isolate FT07026 obtained from Pinery, SA in 2007 was chosen following on from the work of Davidson (2012a) and more recent epidemiology studies of Khani et al. (2016b) and is considered a moderately aggressive isolate. Isolate FT01511 obtained from Riverton, SA in 2015 was chosen to represent a more recent but still moderately to highly aggressive isolate of *P. koolunga*.

For each isolate, eight pots of *P. sativum* cv. Kaspa were planted with four seeds per pot in BioGro™ soil (van Schaik Pty Ltd, SA) and grown for 19–21 days in the CER conditions described in Section 2.3.3 and watered as needed. Seven days after planting the field pea seed, mycelial plugs of each isolate were retrieved from storage at 4 ºC, placed onto potato dextrose agar (PDA, Oxoid™) plates and incubated under 12 h black and fluorescent light/12 h darkness cycle at 22 ºC.

After 12-14 days, conidial suspensions were prepared and adjusted to 1.5×10^5 conidia/mL. Tween 20 (0.01%) was added as a surfactant (Davidson et al. 2012). The suspension of conidia of each isolate was applied to run-off using a hand-held garden sprayer, on eight pots per isolate containing four 21-day old plants. Pots were covered with a single plastic bag for 48 hours to promote humidity and disease development.

At 7 dpi, diseased leaves with symptoms typical of Ascochyta blight were harvested and surface disinfected in a laminar flow cabinet following the method of Davidson et al. (2009a). Leaves with lesions were dipped in 70 % ethanol for 30 s, 1 % sodium hypochlorite for 30 s followed by sterile RO water for 30 s then blotted and left to dry on sterile filter paper in a

laminar flow cabinet. From areas of leaf with lesions, seven segments of approximately 5 × 3 mm were excised and placed in each of eight plates containing acidified PDA (APDA) containing 1 mL of 85 % lactic acid/L of PDA to minimise the risk of bacterial contamination. Plates were incubated under 12 h black and fluorescent light/12 h darkness cycle at 22 ºC for 10 days at which time colonies had mature pycnidia releasing conidia. Mycelial plugs were then taken from colony margins and stored at 4 ºC in Eppendorf tubes containing 1 ml of sterile RO water until ready for use for host range inoculations.

Appendix 2

Naming novel species

To provide a uniform and uniquely Australian theme for naming our novel isolates from Australian native legumes, an appropriate word, or words, from the Indigenous First Australian Peoples language group from where the novel isolates were found was chosen for the species epithet. This decision acknowledges the Australian Indigenous Peoples and languages. Permission to use words was requested from elders or community representatives.

The six novel isolates were from areas represented by four language groups. The Australian Institute of Aboriginal and Torres Strait Islander Studies (AIATSIS) language group map of Australia was used to determine the language group. The Kaurna language representing the Adelaide plains region was used for two isolates, one *Didymella* and one *Neodidymelliopsis* species. Kaurna Warra Karrpanthi (KWK) gave permission to use “tinkyukuku”, which means “leaf disease”. Despite being a most suitable epithet, this could be used for one isolate only due to concerns about potential future homonyms and resulting taxonomic confusion (R. Shivas, 18 October 2019, *pers. comm.*). The second species from the Adelaide plains region was named after the language group itself, namely “Kaurna”.

The Barngarla language group of the Eyre Peninsula provided a name for one novel *Nothophoma* species, for which there were five isolates from different locations in SA. The local community gathered at Port Lincoln in the Eyre Peninsula and proposed the name “garlbi wala warda”, which translates as “leaf”, “fun guy” (together fungi).

In the Murray River region of SA, the local language group covering the Blanchetown area is called Ngayawang. The Ngayawang language is considered extinct. Documents from the mid-1800s recorded local language words, including a reference entitled, “Information respecting the appearance, habits, language, superstitions, government, diseases, warfare of the Aboriginal inhabitants of that part of New Holland known as the South Province

of South Australia". This reference was reported to be contained in correspondence written from Adelaide, dated 4th December 1843 by John Weatherstone, and contained in Papers of the Methodist Missionary Society. These papers were made available by the National Library in Canberra, ACT. Additionally, the book "A vocabulary, and outline of the grammatical structure of the Murray River language: spoken by the natives of South Australia, from Wellington on the Murray, as far as the Rufus", by M. Moorhouse (printed in Adelaide 1846) was made available by the State Library of NSW (Mitchell Library). Both sources were accessed and reviewed. The book by Moorhouse did contain a listing of words, however none was appropriate for a fungal leaf disease epithet. The documents on digitised microfilm from the Methodist Missionary Society Records, Incoming Correspondence, 1812-1889, including many hand-written letters, but no local Aboriginal language words were documented (https://nla.gov.au/nla.obj-742488699/findingaid#nla-obj-756122378). Consequently, Ngayawang and a documented variant of this local language group name, Naiawu, were chosen to acknowledge this Indigenous language group region. As these words for the language groups are in the public domain, an Aboriginal elder employed by the University of Adelaide advised that specific permission was not required.

A Dharawal elder gave permission to use that language from west of Sydney to name one *Didymella* species. The name chosen was "djirang nandiri", which means "leaf spot". Words are referenced in the dictionary of the D'harawal Language with grammatical notes: compiled by Gavin Andrews, Frances Bodkin and Gawaian Bodkin-Andrews (https://dharawalstories.com/dharawal-dictionary).

Appendix 3

Additional Tables and Figures for Chapter 3

Field collection locations

The Gains Research and Development Corporation (GRDC) legume National Variety Trials (NVT) located in field pea growing regions of NSW, SA, and VIC were identified as key locations for initiating field sampling. The NVT site GPS coordinates were obtained from the GRDC NVT website for collections undertaken in 2017 and are shown below in Table I.

Table I. National Variety Trial locations in New South Wales, South Australia and Victoria sampled for *P. koolunga*.

State	Region Name	Nearest Town	GPS Coordinates Latitude	GPS Coordinates Longitude	Sowing Date	Sampling Date
NSW	Mid-West	Grogan near Temora	-34.334497	147.736684	Not provided	09Oct2017
NSW	Mid-West	Lockhart near Boree Creek	-35.24981	146.68161	Not provided	12Oct2017
SA	Mid North	Laura	-33.109021	138.333419	19May2017	15Aug2017
SA	Mid North	Riverton	-34.141393	138.800559	08Jun2017	17Sep2017
SA	South East	Mundulla	-36.32148	140.47519	10May2017	27Aug2017
SA	Yorke Peninsula	Minlaton	-34.786057	137.567787	30May2017	20Aug2017
SA	Yorke Peninsula	Willamulka	-33.924335	137.882340	11May2017	20Aug2017
VIC	Mallee	Birchip	-35.83883	142.77778	08May2017	25Aug2017
VIC	Mallee	Rainbow	-35.94148	142.02707	10May2017	25Aug2017
VIC	Wimmera	Kaniva	-36.368945	141.211146	01Jun2017	26Aug2017
VIC	Wimmera	Tarranyurk	-36.196015	142.036218	24May2017	24Aug2017

Location of sample collection

Table II. GPS coordinates for sites sampled that yielded isolates of interest

Town	Latitude	Longitude
Adelaide Botanic gardens SA	-34.918452	138.611937
Berri SA	-34.24981	140.61873
Blanchetown SA	-34.370149	139.542095
Blinman SA	-31.118769	138.692958
Blyth SA	-33.759455	138.415546
Brown Hill Creek SA	-34.988142	138.628328
Clare SA	-33.866425	138.621368
Kimba SA	-33.138616	136.417484
Mundulla SA	-36.32148	140.47519
Riverton SA	-34.141393	138.800559
Truro SA	-34.407947	139.138514
Urrbrae SA	-34.96781	138.63589
Wudinna SA	-32.985645	135.607143
Longerenong VIC	-36.681282	142.351026
Nhill VIC	-36.31687	141.638196
Rainbow VIC	-35.94148	142.02707
Tarranyurk VIC	-36.196015	142.036218
Boree Creek NSW	-35.24981	146.68161
Cowra NSW	-33.875343	148.645168
Leura NSW	-33.710134	150.368243
Mt Annan NSW	-34.068906	150.76659
Temora NSW	-34.3344497	147.736684

Fig. I Map of south east Australia showing field survey sampling locations based on GPS coordinates shown in Table II

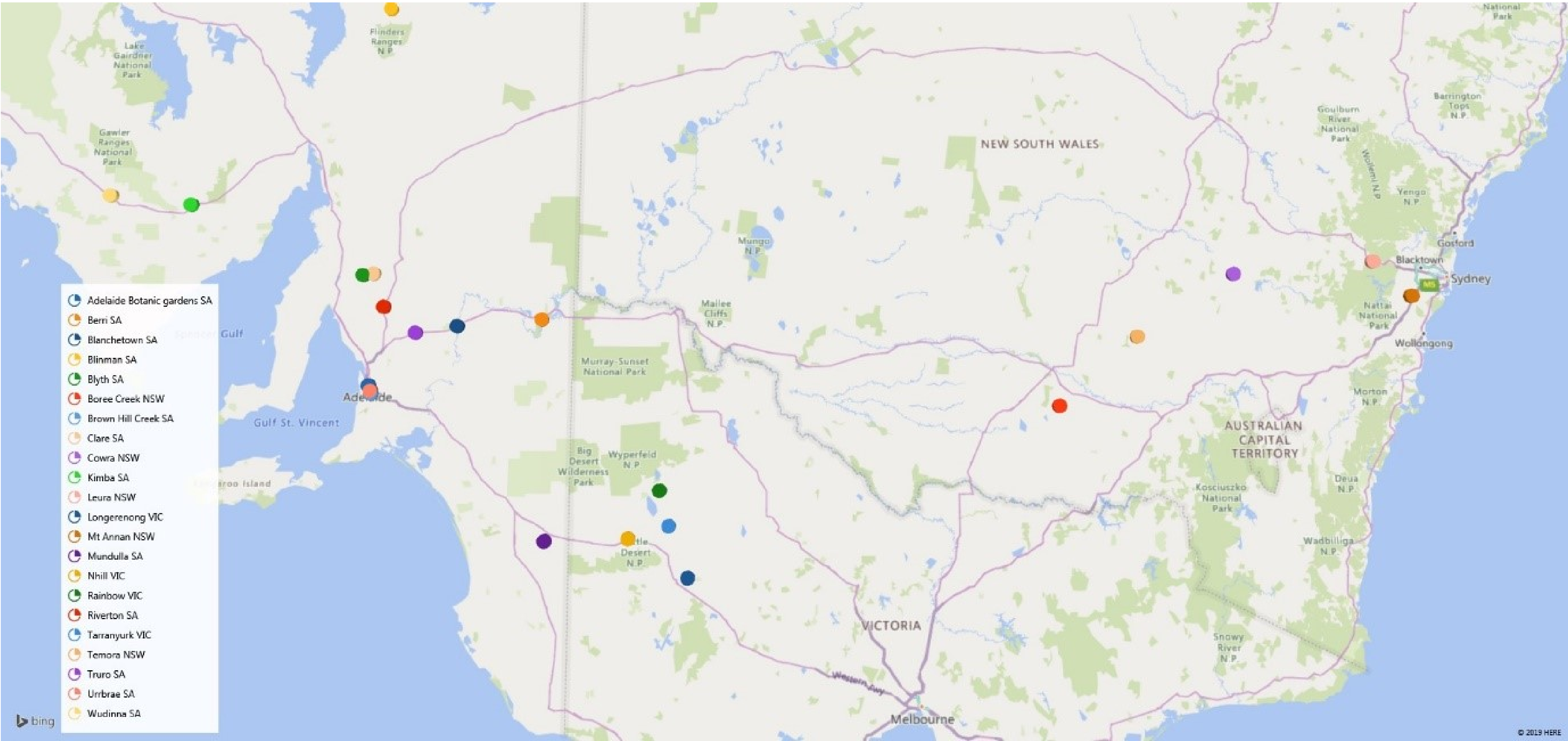

Table III. New host-pathogen associations for *Didymella* species in Australia

Fungal species	Host species	Location	BRIP No in phylogenetic trees (Chapter 3, Fig.1, Appendix 3, Figs II, 111)
Didymella pinodes	*Senna artemisioides*	Berri SA Blanchetown SA Blyth SA Kimba SA Wudinna SA	not included 69581 69593 not included 69596
Didymella pinodes	*Vicia cracca*	Cowra NSW	69593
Didymella lethalis	*Lathyrus tingitanus*	Brownhill Creek, Adelaide, SA	69584

Taxonomic descriptions of *Didymella pinodes* and *Didymella lethalis*

D. pinodes isolate (BRIP 69596) from *S. artemisioides* (see Chapter 3 Fig. 8).

Conidiomata pycnidial, globose to subglobose, 200–300 µm diam, pale brown becoming black, solitary, abundant, scattered within colony, glabrous, non-papillate; ostiole c. 25 µm diam; pycnidial wall composed of textura globulosa, pale brown, cells 5–8 µm diam. *Conidiogenous cells* phialidic, cylindrical, thin-walled, hyaline. *Conidia* medially septate, 7.5–16 × 3.5–5 µm, cylindrical to narrow ellipsoidal, hyaline, thin-walled, straight or slightly curved.

Culture characteristics — Colonies on OA, 37–45 mm diam after 7 d, scant tufted aerial mycelia, olivaceous; reverse olivaceous, two irregular zonate rings.

Colonies on MEA 19–21 mm after 7 d, margin entire, flat grey olivaceous becoming olivaceous black in centre with abundant zonate pycnidia; reverse olivaceous black.

Colonies on PDA, 19–21 mm after 7 d, margin entire, flat grey olivaceous becoming olivaceous black in centre with abundant zonate pycnidia; reverse olivaceous black.

NaOH spot test: negative. Crystals absent.

D. lethalis isolate (BRIP 69584) from *L. tingitanus* (see Chapter 3 Fig. 10)

Conidiomata pycnidial, globose to subglobose, 200–300 µm diam, pale brown becoming black, solitary, abundant, scattered within colony, glabrous, non-papillate; ostiole c. 25 µm diam; pycnidial wall composed of textura globulosa, pale brown, cells 5–8 µm diam. *Conidiogenous cells* phialidic, cylindral, thin-walled, hyaline. *Conidia* medially septate, 8–15 × 2.5–4.0 µm, cylindrical to narrow ellipsoidal, hyaline, thin-walled, straight or slightly curved.

Culture characteristics — Colonies on OA, 40–44 mm diam after 7 d, margin entire, flat with scattered tufted aerial mycelium, dark mouse grey; reverse fuscous.

Colonies on MEA, 45–50 mm diam after 7 d, margin irregular, flat to felty, olivaceous with abundant scattered pycnidia; reverse dark olivaceous.

Colonies on PDA, 36–40 mm after 7 d, margin irregular, felty, fuscous black; reverse fuscous black.

NaOH spot test: negative. Crystals absent.

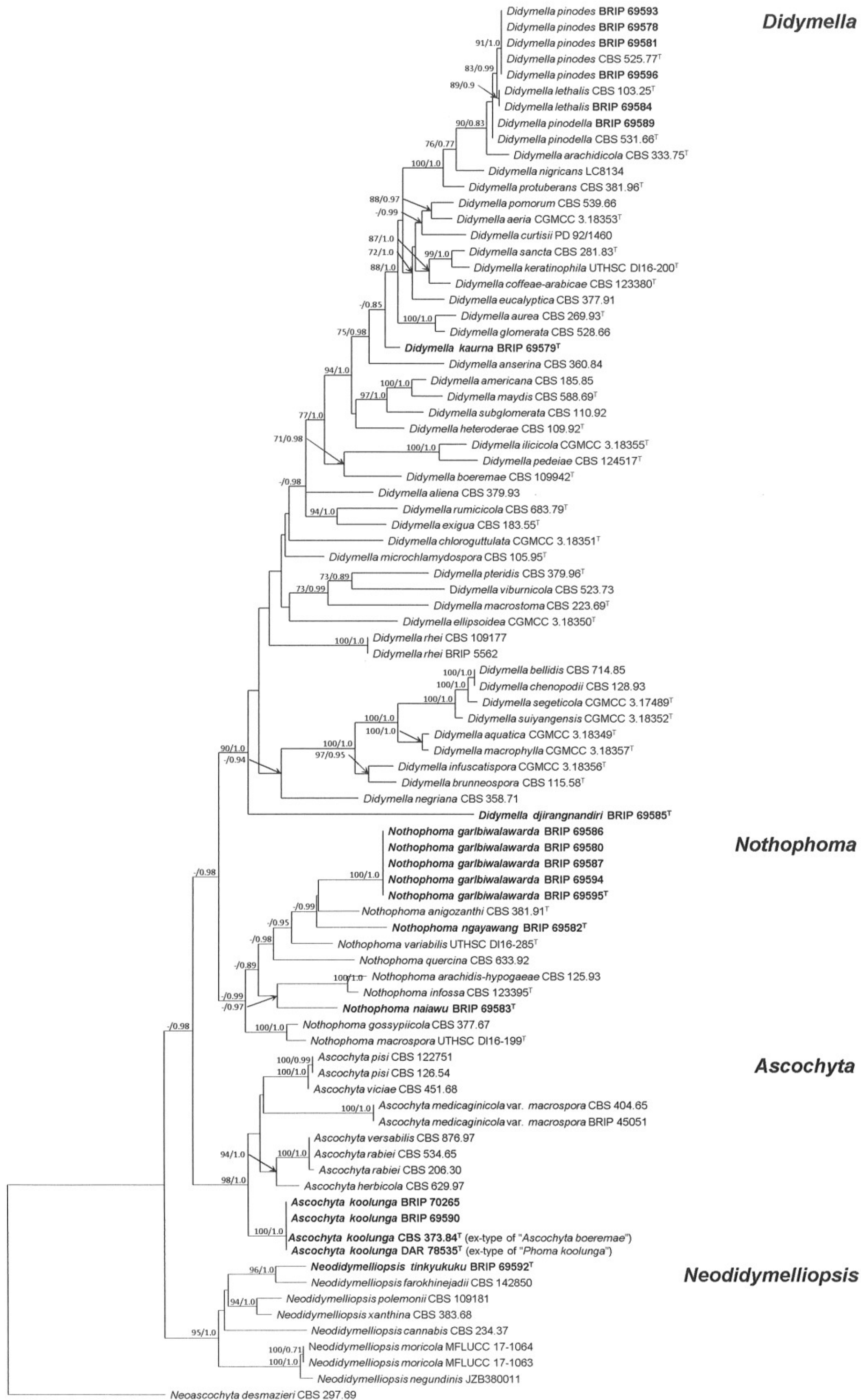
Didymella
Didymella pinodes BRIP 69593
Didymella pinodes BRIP 69578
Didymella pinodes BRIP 69581
Didymella pinodes CBS 525.77T
Didymella pinodes BRIP 69596
Didymella lethalis CBS 103.25T
Didymella lethalis BRIP 69584
Didymella pinodella BRIP 69589
Didymella pinodella CBS 531.66T
Didymella arachidicola CBS 333.75T
Didymella nigricans LC8134
Didymella protuberans CBS 381.96T
Didymella pomorum CBS 539.66
Didymella aeria CGMCC 3.18353T
Didymella curtisii PD 92/1460
Didymella sancta CBS 281.83T
Didymella keratinophila UTHSC DI16-200T
Didymella coffeae-arabicae CBS 123380T
Didymella eucalyptica CBS 377.91
Didymella aurea CBS 269.93T
Didymella glomerata CBS 528.66
Didymella kaurna BRIP 69579T
Didymella anserina CBS 360.84
Didymella americana CBS 185.85
Didymella maydis CBS 588.69T
Didymella subglomerata CBS 110.92
Didymella heteroderae CBS 109.92T
Didymella ilicicola CGMCC 3.18355T
Didymella pedeiae CBS 124517T
Didymella boeremae CBS 109942T
Didymella aliena CBS 379.93
Didymella rumicicola CBS 683.79T
Didymella exigua CBS 183.55T
Didymella chloroguttulata CGMCC 3.18351T
Didymella microchlamydospora CBS 105.95T
Didymella pteridis CBS 379.96T
Didymella viburnicola CBS 523.73
Didymella macrostoma CBS 223.69T
Didymella ellipsoidea CGMCC 3.18350T
Didymella rhei CBS 109177
Didymella rhei BRIP 5562
Didymella bellidis CBS 714.85
Didymella chenopodii CBS 128.93
Didymella segeticola CGMCC 3.17489T
Didymella suiyangensis CGMCC 3.18352T
Didymella aquatica CGMCC 3.18349T
Didymella macrophylla CGMCC 3.18357T
Didymella infuscatispora CGMCC 3.18356T
Didymella brunneospora CBS 115.58T
Didymella negriana CBS 358.71
Didymella djirangnandiri BRIP 69585T
Nothophoma
Nothophoma garlbiwalawarda BRIP 69586
Nothophoma garlbiwalawarda BRIP 69580
Nothophoma garlbiwalawarda BRIP 69587
Nothophoma garlbiwalawarda BRIP 69594
Nothophoma garlbiwalawarda BRIP 69595T
Nothophoma anigozanthi CBS 381.91T
Nothophoma ngayawang BRIP 69582T
Nothophoma variabilis UTHSC DI16-285T
Nothophoma quercina CBS 633.92
Nothophoma arachidis-hypogaeae CBS 125.93
Nothophoma infossa CBS 123395T
Nothophoma naiawu BRIP 69583T
Nothophoma gossypiicola CBS 377.67
Nothophoma macrospora UTHSC DI16-199T
Ascochyta
Ascochyta pisi CBS 122751
Ascochyta pisi CBS 126.54
Ascochyta viciae CBS 451.68
Ascochyta medicaginicola var. macrospora CBS 404.65
Ascochyta medicaginicola var. macrospora BRIP 45051
Ascochyta versabilis CBS 876.97
Ascochyta rabiei CBS 534.65
Ascochyta rabiei CBS 206.30
Ascochyta herbicola CBS 629.97
Ascochyta koolunga BRIP 70265
Ascochyta koolunga BRIP 69590
Ascochyta koolunga CBS 373.84T (ex-type of "Ascochyta boeremae")
Ascochyta koolunga DAR 78535T (ex-type of "Phoma koolunga")
Neodidymelliopsis
Neodidymelliopsis tinkyukuku BRIP 69592T
Neodidymelliopsis farokhinejadii CBS 142850
Neodidymelliopsis polemonii CBS 109181
Neodidymelliopsis xanthina CBS 383.68
Neodidymelliopsis cannabis CBS 234.37
Neodidymelliopsis moricola MFLUCC 17-1064
Neodidymelliopsis moricola MFLUCC 17-1063
Neodidymelliopsis negundinis JZB380011
Neoascochyta desmazieri CBS 297.69

Fig. II Phylogenetic tree inferred from a Maximum likelihood analysis based on *rpb*2 sequence alignment of 18 isolates representing Didymellacea. RAxML boostrap values (bs) greater than 70% and Bayesian posterior probabilities (pp) greater than 0.7 are given at the nodes (bs/pp). Novel taxa and combination introduced in this study are in bold. Novel taxa and combination introduced in this study are in bold. The outgroup is *Neoascochyta desmazieri* strain CBS 297.69.

BRIP 69595
Didymella pinodes CBS 525.77
Didymella lethalis CBS 103.25
BRIP 69578
BRIP 69589
BRIP 69593
BRIP 69584
BRIP 69581
Didymella arachidicola CBS 333.75
Didymella pinodella CBS 531.66
Didymella viburnicola CBS 523.73
Didymella pteridis CBS 379.96
Didymella macrostoma CBS 223.69
Didymella protuberans CBS 381.96
Didymella nigricans PD 77/919
Didymella aliena CBS 379.93
Didymella heteroderae CBS 109.92
Didymella segeticola CGMCC 3.17489
Didymella suiyangensis CGMCC 3.18352
Didymella bellidis CBS 714.85
Didymella brunneospora CBS 115.58
Didymella infuscatispora CGMCC 3.18356
Didymella macrophylla CGMCC 3.18357
Didymella aquatica CGMCC 3.18349
Didymella ilicicola CGMCC 3.18355
Didymella pedeiae CBS 124517
Didymella exigua CBS 183.55
Didymella rhei CBS 109177
Didymella rhei BRIP 5562
Didymella chloroguttulata CGMCC 3.18351
Didymella subglomerata CBS 110.92
Didymella americana CBS 185.85
Didymella maydis CBS 588.69
Didymella anserina CBS 360.84
Didymella sinensis LC 5246
BRIP 69579
Didymella pomorum CBS 539.66
Didymella microchlamydospora CBS 105.95
Didymella curtisii PD 92/1460
Didymella keratinophila UTHSC DI16-200
Didymella eucalyptica CBS 377.91
Didymella negriana CBS 358.71
Didymella sancta CBS 281.83
Didymella coffeae-arabicae CBS 123380
Nothophoma variabilis UTHSC DI16-285
BRIP 69592
Neodidymelliopsis ranunculi MFLUCC 13-0490
Neodidymelliopsis longicolla CBS 382.96
Neodidymelliopsis farokhinejadii CBS 142850
Neodidymelliopsis achlydis CBS 256.77
Neodidymelliopsis xanthina CBS 383.68
Neodidymelliopsis polemonii CBS 109181
Neodidymelliopsis negundinis JZB380020
Neodidymelliopsis negundinis JZB380011
Neodidymelliopsis cannabis CBS 234.37
Neodidymelliopsis moricola MFLUCC 17-1064
Neodidymelliopsis moricola MFLUCC 17-1063
Didymella boeremae CBS 109942
Didymella aeria CGMCC 3.18353
Ascochyta fabae CBS 524.77
Ascochyta viciae CBS 451.68
Ascochyta lentis CBS 370.84
Ascochyta pisi CBS 126.54
Ascochyta syringae CBS 545.72
Ascochyta pisi CBS 122751
Ascochyta viciae-pannonicae CBS 254.92
Ascochyta medicaginicola var. macrospora CBS 404.65
Ascochyta medicaginicola var. medicaginicola CBS 316.90
Ascochyta medicaginicola var. macrospora BRIP 45051
Ascochyta nigripycnidia CBS 116.96
Ascochyta phacae CBS 184.55
Ascochyta rabiei CBS 206.30
Ascochyta rabiei CBS 534.65
Ascochyta rabiei CBS 237.37
Ascochyta herbicola CBS 629.97
Phoma koolunga DAR 78535
BRIP 69590
Ascochyta boeremae CBS 373.84
BRIP 70265
Nothophoma macrospora UTHSC DI16-199
BRIP 69583
BRIP 69585
Didymella aurea CBS 269.93
Didymella rumicicola CBS 683.79
Didymella glomerata CBS 528.66
Nothophoma quercina CBS 633.92
Nothophoma infossa CBS 123395
Nothophoma arachidis-hypogaeae CBS 125.93
Nothophoma anigozanthi CBS 381.91
BRIP 69587
BRIP 69594
BRIP 69596
BRIP 69586
BRIP 69580
Nothophoma gossypiicola CBS 377.67
Didymella ellipsoidea CGMCC 3.18350
Ascochyta versabilis CBS 876.97
BRIP 69582
Didymella chenopodii CBS 128.93
Neoascochyta desmazieri CBS 297.69
-/0.86
82/0.99
-/0.85
-/0.88
81/0.91
-/0.96
92/1.0
100/1.0
-/0.76
-/1.0
76/0.86
93/1.0
94/-
90/0.99
71/0.96
71/0.96
100/1.0
81/0.91
100/1.0
-/0.79
-/0.81
0.03

Fig. III Phylogenetic tree inferred from a Maximum likelihood analysis based on ITS sequence alignment of 18 isolates representing Didymellacea. RAxML boostrap values (bs) greater than 70% and Bayesian posterior probabilities (pp) greater than 0.7 are given at the nodes (bs/pp). Novel taxa and combination introduced in this study are in bold. Novel taxa and combination introduced in this study are in bold. The outgroup is *Neoascochyta desmazieri* strain CBS 297.69.

Additional isolates of *Nothophoma garlbiwalawarda*

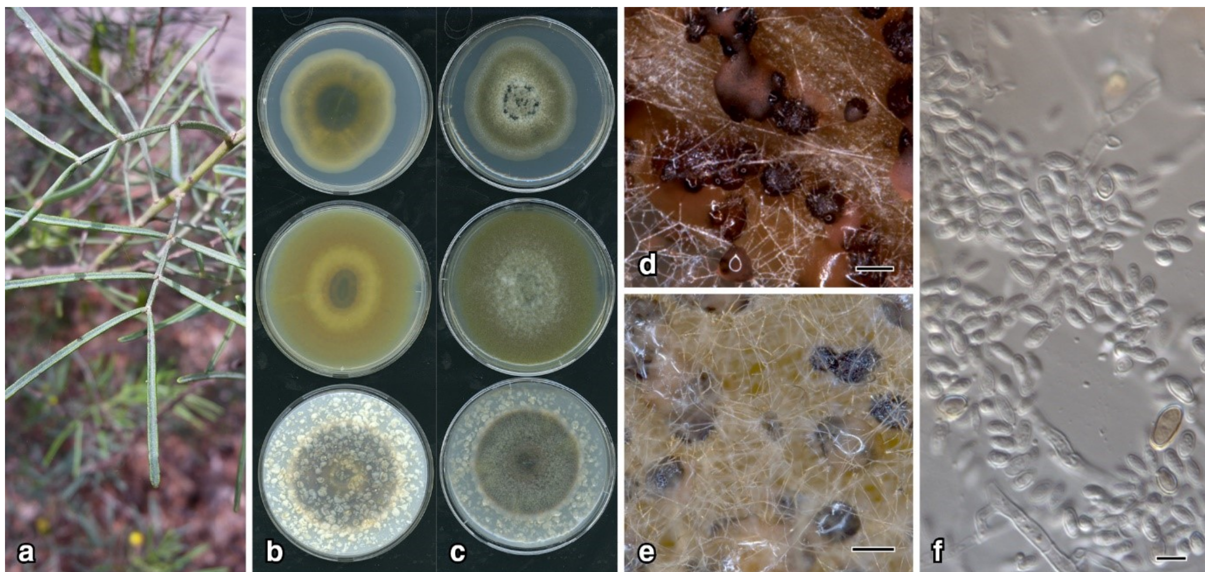

Fig. IV *Nothophoma garlbiwalawarda* **a** Pin-prick leaf spots on *Senna artemisioides* from Urrbrae, Adelaide, SA; **b** 14-d old colonies, top to bottom, on PDA, MEA, OA lower surface, **c** upper surface; **d** pycnidia on CLA; **e** pycnidia and pycnidial ooze on OA; **f** conidia. Scale bars: d-e = 200 µm; f = 10 µm.

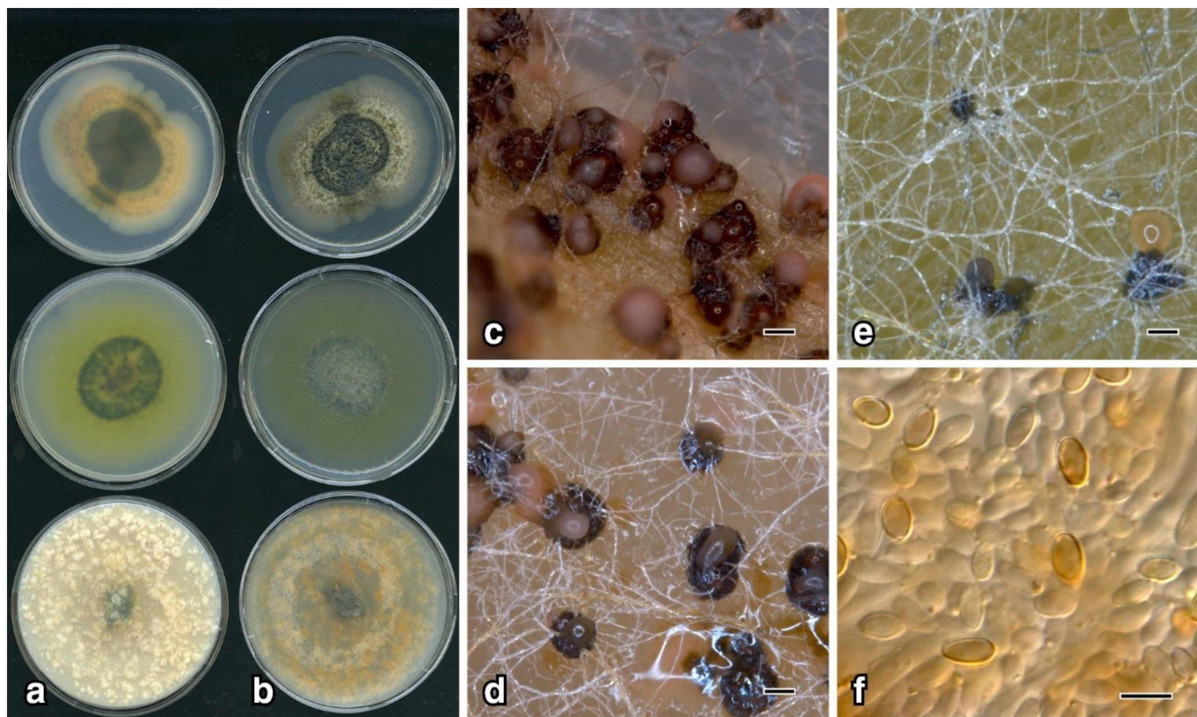

Fig. V *Nothophoma garlbiwalawarda* from *Senna artemisioides* collected at Berri, SA; **a** 12-d old colonies, top to bottom, on PDA, MEA, OA lower surface, **b** upper surface; **c** pycnidia on CLA; **d** pycnidia and pycnidial ooze on OA; **e** pycnidia and pycnidial ooze on PDA; **f** conidia. Scale bars: c-e = 200 µm; f = 10 µm.

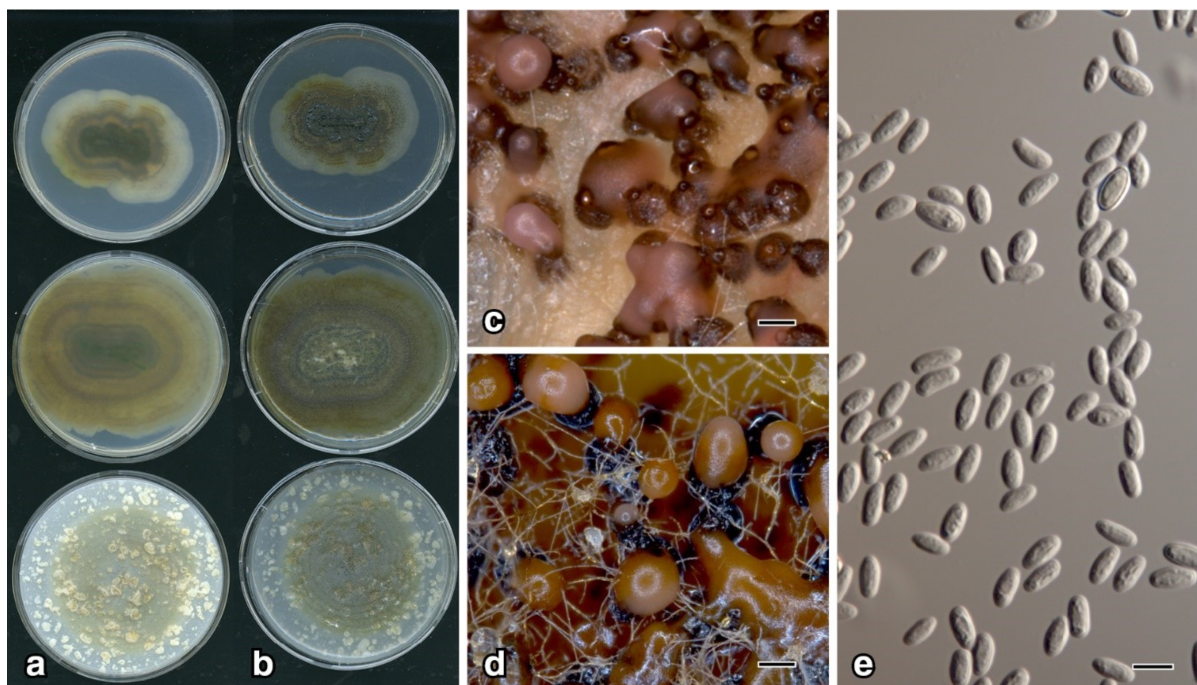

Fig. VI *Nothophoma garlbiwalawarda* from *Senna artemisioides* collected at Berri, SA; **a** 12-d old colonies, top to bottom, on PDA, MEA, OA lower surface, **b** upper surface; **c** pycnidia on CLA; **d** pycnidia and pycnidial ooze; **e** conidia. Scale bars: c-d = 200 µm; e = 7 µm.

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