Epidemiology and management of ascochyta blight of field pea (*Pisum sativum*) in South Australia

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ABSTRACT

Ascochyta blight disease (synonym: blackspot) of field pea has worldwide distribution and regularly causes AUD$25 million loss per annum in Australian field pea (*Pisum sativum*) crops. This study provides new information on the causal pathogens and management strategies to reduce loss from this disease.

Research involving sowing dates, genotypes and fungicide treatments was conducted to identify optimal management strategies. Earlier sowing generally resulted in higher yield except when ascochyta blight was severe. Yield response to fungicide application varied with disease severity, sowing date and genotype. The optimum sowing period was within a week of the first autumn rains in low rainfall regions and 3 weeks after the first autumn rains in medium and medium - high rainfall regions. Earlier flowering genotypes were the highest yielding particularly when sown early and subjected to strategic fungicide applications.

The pathogen, *Phoma koolunga*, was recognised for the first time as a component of the ascochyta blight disease complex in southern Australia. The species was described morphologically. Sequences of the internal transcribed spacer region were distinct from those of the accepted causal pathogens of ascochyta blight of field pea *viz*. *Didymella pinodes*, *Phoma medicaginis* var. *pinodella* and *Ascochyta pisi*. Symptoms on field pea seedlings caused by *P. koolunga* were indistinguishable from those caused by *D. pinodes*, other than a 24 h delay in manifestation of symptoms.

P. koolunga was detected across field pea cropping soils in South Australia but rarely from other Australian states while *D. pinodes* plus *P. medicaginis* var. *pinodella* were widespread. The quantity of DNA of these pathogens detected in soils was positively correlated with ascochyta blight lesions in a pot bioassay. Soil-borne inoculum gradually decreased in the 3 years following a field pea crop. DNA tests and pathogen isolation from naturally infected field pea plants showed *P. koolunga* to be an important component of the

disease complex in South Australia. *P. koolunga* and *D. pinodes* were equally responsible for disease symptoms, while *P. medicaginis* var. *pinodella* had a minor role in the disease complex.

Interaction between *D. pinodes, P. medicaginis* var. *pinodella* and *P. koolunga* was investigated in controlled conditions. Colony diameter of the former was reduced on potato dextrose agar (PDA) amended with filtrate from broth cultures of *P. koolunga*, as was colony diameter of *D. pinodes* on PDA amended with filtrate from *P. medicaginis* var. *pinodella* or *D. pinodes*. This effect was shown to be fungistatic rather than fungicidal. When co-inoculated onto leaves on field pea plants, or onto excised leaf discs, either the quantity of DNA of *D. pinodes* and of *P. medicaginis* var. *pinodella,* or the mean lesion diameter of these pathogens, was significantly reduced when co-inoculated with *P. koolunga*. *P. koolunga* was not influenced by co-inoculation. *D. pinodes* demonstrated self-antagonism.

D. pinodes is considered the principal pathogen of concern in this complex. This study further investigated the relationship between ascospore numbers of *D. pinodes* at sowing and disease at the end of the season. Ascospores released from stubble infested with ascochyta blight were counted periodically in a wind tunnel. A model was developed to predict disease severity in relation to ascospore numbers, distance from infested field pea stubble, and rainfall. The model was validated with an independent dataset. A threshold level of ascospores of *D. pinodes* was identified above which disease did not increase.

The findings from this study have been incorporated into management recommendations for field pea in southern Australia. Growers are encouraged to manipulate sowing dates according to the temporal release of ascospores, and select a cultivar that has the best agronomic yield potential for the sowing date, and to use fungicide strategically. The recommendation also emphasises field selection based on commercial testing for the presence of soil-borne inoculum of *D. pinodes*, *P. medicaginis* var. *pinodella* and *P. koolunga*.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma 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.

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STATEMENT OF THE CONTRIBUTIONS TO JOINTLY AUTHORED PAPERS

1. Davidson JA, Hartley D, Priest M, Krysinska-Kaczmarek M, Herdina, McKay A, Scott ES (2009) A new species of *Phoma* causes ascochyta blight symptoms on field peas (*Pisum sativum)* in South Australia. *Mycologia* **101**, 120-128 [published manuscript].

Presented in Chapter 2 Author contributions: JAD designed and conducted the research experiments, analysed the data, and drafted and constructed the manuscript. DH identified genetic sequences and conducted evolutionary analysis and contributed to the manuscript, MP contributed the taxonomic description of *P. koolunga*, MKK provided technical assistance and support of experiments, H and AM conducted DNA analyses and contributed to the manuscript, ESS supervised research, contributed to the research ideas and design, and the editing of the manuscript.

2. McMurray LS, Davidson JA, Lines MD, Leonforte A, Salam MU (2011) Combining management and breeding advances to improve field pea (*Pisum sativum* L.) grain yields under changing climatic conditions in south-eastern Australia. *Euphytica* **180**, 69-88 [published manuscript].

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Each of these manuscripts is displayed in this thesis in either published or submitted form according to the instructions to author of the specific journal.

This thesis has been prepared according to the University of Adelaide's specifications for 'PhD by publications' format.

The following authors agree that the statement of the contributions of jointly authored papers accurately describes their contribution to research manuscripts 1 to 5 and give consent to their inclusion in this thesis.

Davidson JA ……

Hartley D ……

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ABBREVIATIONS

ANOVA	analysis of variance
AUD	Australian Dollar
AUDPC	area under disease progress curve
AWS	automatic weather station
BLUP	best linear unbiased prediction
CER	controlled environment room
CSIRO	Commonwealth Scientific and Industrial Research Organisation
cv.	cultivar
cvs	cultivars
DAI	days after inoculation
DAS	days after sowing
diam	diameter
DiseaseAugSoil	contribution of ascochyta blight from soil inoculum, measured at the end of August
DNA	deoxyribonucleic acid
DpPmp	*Didymella pinodes* plus *Phoma medicaginis* var. *pinodella*
Early MM	two applications of mancozeb (at 4 nodes and at flowering)
ELISA	enzyme-linked immuno-sorbent assay
G3PD	glyceraldehyde – 3 – phosphate dehydrogenase
GS	growth stage
IAD	internode area diseased
IGS	intergenic spacer
ITS	internal transcribed spacer
LAD	leaf area diseased
LSD	least significant difference
L rainfall	low rainfall
M rainfall	medium rainfall
M fungicide	application of mancozeb at 9 nodes
MH rainfall	medium to high rainfall
MM fungicide	two applications of mancozeb (at 9 nodes and at flowering)
NSW	New South Wales
NUV	near ultraviolet
PCR	polymerase chain reaction
PDA	potato dextrose agar (full strength)
p.i.	post inoculation
QTL	quantitative trait loci
RainAS	rain summed for August and September
RainWinter	rain summed from sowing date to end of July
RAPD	random amplified polymorphic DNA
RDTS	Root Disease Testing Service
Rec	recommended sowing date
Rec-2	sowing date 2 weeks earlier than recommended

Rec-4	sowing date 4 weeks earlier than recommended
REML	residual maximum likelihood
RFLP	restriction fragment length polymorphism
RIE	radiation interception efficiency
RO	reverse osmosis
RUE	radiation use efficiency
SA	South Australia
SARDI	South Australian Research and Development Institute
SD	seed dressing (P-Pickel T®)
%Spores	% of ascospores remaining on stubble estimated by G1 Blackspot Manager
SporesInit	initial number of ascospores
TOS	time of sowing
Vic.	Victoria
WA	Western Australia
WAS	weeks after sowing

Chapter 1.

Introduction and Review of Literature

CHAPTER 1

Introduction and Review of Literature

1.1 Introduction

Ascochyta blight (synonym: blackspot) of field pea (*Pisum sativum* L.) has a world-wide distribution, including temperate regions such as Europe, North America, Australia and New Zealand, where field pea are grown in large areas, and in the sub-tropical areas of Africa, and Central and South America (Basu *et al*. 1973, Beasse *et al*. 1999, Bretag and Ramsey 2001). In Australia, very few field pea crops are free from this disease and yield losses of 15 - 75 % occur regularly. Most Australian field pea crops are infected soon after planting but it is the rapid disease spread in spring associated with rainfall that causes the greatest damage (Bretag 1991, 1995a, Bretag and Ramsey 2001, Wroth 1998b). Ascochyta blight is considered the major disease of field pea in Australia (Bretag *et al.* 1995b, 2006, Davidson and Ramsey 2000) and resistance is one of the primary goals of the Pulse Breeding Australia (PBA) Field Pea Breeding Program (Ali *et al.* 1994, Siddique and Sykes 1997).

Three fungal pathogens, existing independently of each other, are recognised as the causal agents of this disease. *Didymella pinodes* (synonym: *Mycosphaerella pinodes*) (Berk. & Blox) Vestergr. is considered the most aggressive of these (Kraft *et al.* 1998b). In one comparative study in Canada, this pathogen caused 45 % yield loss, and the other pathogens, *Phoma medicaginis* Marlbr. & Roum. var. *pinodella* (synonym: *Ascochyta pinodella* Jones) and *Ascochyta pisi* Lib., individually caused 25 % and 11 % yield loss (Wallen 1964). *P. medicaginis* var. *pinodella* can cause severe foot-rot, leading to a reduction in plant size (Hare and Walker 1944, Knappe and Hoppe 1995, Onfroy *et al*. 1999). *A. pisi* has a very limited role in ascochyta blight of field pea in Australian conditions (Ali and Dennis 1992, Barbetti *et al*. 1989) and a reduction in the population of *A. pisi* was also recorded in Canada associated with the introduction of resistant cultivars (Wallen *et al*. 1967a). However, recently *A. pisi*

has, inexplicably, been detected more frequently in Canada (Warkentin *et al.* 2012) and the Czech Republic (Dostalova *et al*. 2012). A fourth fungal pathogen, initially identified as *Macrophomina phaseolina* (Ali and Dennis 1992), but subsequently classified as an unidentified species of the *Phoma* genus (Dr. Hans de Gruyter, Plant Protection Services, Netherlands, personal communication, 13th July 2006), has been associated with ascochyta blight lesions on field pea in South Australia. It is not easy to distinguish between symptoms caused by the four ascochyta blight pathogens (Kraft *et al*. 1998b, Linford and Sprague 1927). While they exist independently of each other, they are regularly found together on individual plants and all are generally considered to cause a single disease (Hare and Walker 1944, Linford and Sprague 1927). The pathogens infect all above-ground parts of the field pea plant as well as the crown below ground level. Symptoms consist of necrotic spots that coalesce into large lesions on stems, leaves and pods, and root rot may also occur (Kraft *et al.* 1998b). Severe infection can lead to seedling death (Wallen 1974, Xue *et al.* 1997). The pathogens can be carried on seed, may be transmitted through the soil and air, or survive in infested crop residues (Ali *et al*. 1982, Ali and Dennis 1992, Carter and Moller 1961, Wallen and Jeun 1968).

No single gene or major gene resistance to ascochyta blight in field pea has been found, despite extensive searches in the gene pool, in particular for resistance to *D. pinodes* (Kraft *et al*. 1998a). Foliar fungicides are usually effective but uneconomic (Davidson and Kimber 2007, Siddique and Sykes 1997, Warkentin *et al.* 1996). Consequently, disease control relies upon management strategies, such as not planting field pea crops adjacent to field pea stubble (Bretag 1991, Bretag *et al.* 2006, Hawthorne *et al.* 2011, McDonald and Peck 2009, Peck *et al*. 2001), rotations of at least 4 years between field pea crops and, in Australia, delayed sowing of field pea crops, to 3 - 4 weeks after the autumn rains (Bretag *et al*. 2000, Davidson and Kimber 2007). However, field pea yields may be compromised by later sowing and losses may be greater than those brought about by ascochyta blight (Bretag *et al*. 2000). This is

particularly important in recent seasons when springs have been hotter and drier than long term average (SILO 2010). Hence, field pea crops need to be sown at a date that minimises ascochyta blight infection without compromising the yield. The success of these practices requires an understanding of the epidemiology of the pathogens and the interaction of the disease with environmental factors. The role of the individual pathogens in the ascochyta complex also needs to be understood in order to develop successful management practices that target all the pathogens involved in this disease.

1.2 Field pea production in South Australia

The field pea plant is an annual trailing plant with tendrils which it uses to climb on supporting structures. Modern cultivars are semi-leafless (*afila*), in which leaftlets are replaced by tendrils that interlink and support each other, resulting in an upright growth habit, increased stem stiffness and improved resistance to lodging. Plants with the upright growth habit are easier to harvest and losses during harvest are less than in the conventional trailing types. The different phenotypes of the conventional and semi-leafless cultivars appear to have no impact on severity of ascochyta blight (Bretag and Brouwer 1995).

In southern Australia, the field pea crop is sown in May or June after the autumn rains and harvested from October to December, depending on the seasonal and regional conditions. In the northern hemisphere, spring-grown cultivars of field pea are sown in March and harvested in August, while the winter-grown cultivars, sown in September and harvested in June, need to be cold tolerant to survive the freezing winter temperatures in these regions (Le May *et al.* 2005). The winter-grown field pea grows vegetatively during winter and flowers and produces seeds as day length increases. Growth is indeterminate and additional reproductive nodes are produced after flowering has begun (Marx 2001).

The cultivars currently grown in southern Australia are Parafield, a conventional type, commercialised in 1999, with an average yield of 2.57 t ha^{-1}, and Kaspa, an erect semi-leafless type commercialised in 2001, which generally yields 7 % more than Parafield. Kaspa

is more commonly grown in the medium to high rainfall zones (400 - 500 mm per annum) of South Australia while Parafield is more suited to the lower rainfall areas (350 - 400 mm per annum), as well as regions prone to moisture stress during flowering and podding. Parafield is also preferred in areas prone to bacterial blight due to the higher susceptibility of Kaspa to this disease (McMurray 2006). The PBA Field Pea Breeding Program has developed lines for the low rainfall region that have earlier and longer flowering periods than Kaspa but with similar architecture. Optimal disease management practices for these new cultivars need to be developed.

From the late 1950s until 1970s a constant area of 12,000 ha of field pea was grown in South Australia and this was the only leguminous crop in the farming system. Field pea filled a valuable role in building soil fertility, which is still an important factor in the popularity of this crop today. The average yield was 0.95 t ha^{-1} (Anon. 1978). In the early years of field pea production in South Australia, early sowing was recommended to increase yields even though ascochyta blight was known to cause complete failure of some crops (McAuliffe and Webber 1962). The link between early sowing and ascochyta blight severity was possibly not widely understood at that time although research on the role of stubble-borne inoculum and resulting ascospores had been published (Carter and Moller 1961, Carter 1963).

There was a large increase in field pea production in South Australia from 40,000 ha in 1984 to over 140,000 ha by 1995 (McDonald 1995, McMurray and Seymour 2005, Siddique and Sykes 1997). This increase was attributed to improved management, in particular the use of post-emergent herbicides, improved cultivars and expanded markets (McDonald 1995), such as India, which imports half of the Australian production (Siddique and Sykes 1997). Average yield rose to approximately 1.5 t ha^{-1} by 1986, due to improved management, but dropped to 1.25 t ha^{-1} by 1993 (McDonald 1995). This drop was attributed to disease and the depletion of soil nutrients under the more intense cropping regime (Davidson and Ramsey 2000, McDonald 1995, McDonald and Peck 2009, Peck and

McDonald 1998). Following the introduction of canola and alternative high value pulse crops such as faba beans, lentils and occasionally chickpeas into the cropping system, the rotation in more recent years has dropped from one field pea crop in 3 years to one in 5 or 6 years (Peck and McDonald 2001).

The production area has stabilised in South Australia, to approximately 110,000 ha in 2011, yielding 1.4 t ha^{-1}. This is nearly half of the total production of field pea in Australia, at 243,500 ha in 2011 (Pulse Australia 2011). Sowing areas in other Australian states in 2011 were 38,000 ha (1.1 t ha^{-1}) in Victoria, New South Wales 40,500 (1.5 t ha^{-1}) and Western Australia 55,000 ha (0.95 t ha^{-1}) (Pulse Australia 2011). The comparatively low average yields in South Australia, Victoria and Western Australia reflect the extension of this crop into the lower rainfall areas (McDonald 1995, McMurray and Seymour 2005).

1.3 Ascochyta blight pathogens

1.3.1 *Didymella pinodes*

D. pinodes (Figure 1) is the homothallic perfect stage of *Ascochyta pinodes* (Kraft *et al*. 1998b). The pathogen has been isolated from *P. sativum*, *Lathyrus*, *Phaseolus* and *Vicia* spp. (Punithalingham and Holliday 1972b). It was originally named *Mycosphaerella pinodes* (Punithalingham and Holliday 1972b) but Peever *et al*. (2007) proposed that *Didymella pinodes* be used since DNA sequences of the internal transcribed spacer (ITS) region clustered with *Didymella* species and not with well characterised *Mycosphaerella* species.

In general, colonies on culture media are light to dark grey, with pseudothecia and pycnidia initially distributed along the radii of mycelium growing out from the central point. After 20 - 30 mm of growth, the pseudothecia and pycnidia become arranged in concentric rings in response to a 12 h photoperiod (Onfroy *et al*. 1999). Pycnidium production is increased with light and decreased at low temperatures (Hare and Walker 1944). On stems, pycnidia are 100 - 200 μm in diameter. Conidia are hyaline, 1 or occasionally 2 septate,

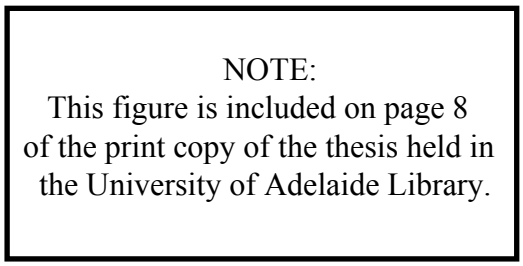


Figure 1.
Conidial and ascosporic stages of *Didymella pinodes* (Berk. & Blox.) Vestrergr.
A. Vertical section of pseudothecium; **B**. ascus; **C**, ascospores; **D**, vertical section of pycnidium; **E**, part of the pycnidial wall and conidiophores; **F**, conidia (reprinted from Punithalingham and Holliday 1972b).

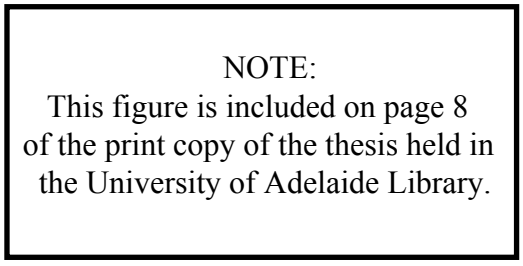


Figure 2.
Conidial and chlamydosporic stages of *Phoma medicaginis* Malbr. & Roum. var. *pinodella* (Jones) Boerema.
A, vertical section of pycnidium; **B**, conidiophores and conidia; **C**, conidia;
D, chlamydospores.
(reprinted from Punithalingham and Gibson 1976).

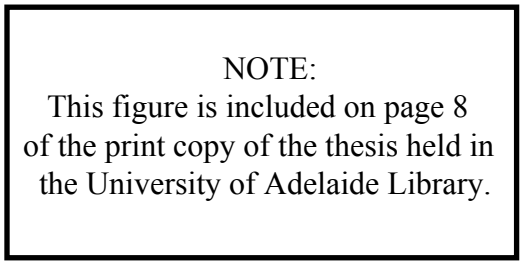


Figure 3.
Conidial stage of *Ascochyta pisi* Lib.
A, Vertical section of pycnidium; **B**, part of the pycnidial wall and conidiophores;
C, conidiophores and conidia; **D**, conidia. (reprinted from Punithalingham and Holliday 1972a).

slightly constricted at the septum and 8 - 16 x 3 - 4.5 µm (Punithalingham and Holliday 1972b). On V8 juice agar over 97 % of the conidia are bicellular (Onfroy *et al.* 1999*). D. pinodes* produces pseudothecia that are dark brown, globose with papillate ostioles and 90 x 180 µm in diameter. The 8-spored asci are 50 - 80 x 10 - 15 µm and the ascospores are hyaline, two-celled, constricted at the septum and are 4 - 8 x 12 - 18 µm (Punithalingham and Holliday 1972b). As a general rule, pseudothecia will develop on poor or minimal media whereas pycnidia are more likely to be produced on highly nutritive media, though significant variability occurs among strains (Hare and Walker 1944, Roger and Tivoli 1996a). *D. pinodes* may produce pseudothecia on malt agar, Mathur's agar medium, oatmeal agar medium and V8 juice medium (Onfroy *et al.* 1999). The most favourable temperature for development and maturation of pseudothecia was 16 ºC, with numbers decreasing at 20 ºC, rare at 24 ºC and 28 ºC, and non-existent at 30 ºC. From 12 ºC to 4 ºC, the numbers of pseudothecia produced were the same but the time to maturation increased from 35 to 100 days at the lower temperature. At 16 ºC abundant pseudothecia matured in 25 - 30 days (Hare and Walker 1944). Chlamydospores are also produced in culture, either singly or in small chains (Punithalingham and Holliday 1972b).

1.3.2 *Phoma medicaginis* var. *pinodella*

This pathogen (Figure 2) was originally named *Ascochyta pinodella* Jones (Bretag *et al.* 2006) but was later moved to the genus *Phoma* and, because its morphology was similar to *Phoma medicaginis* Malbr. & Roum., it was renamed *Phoma medicaginis* (Malbr. & Roum.) var. *pinodella* (Jones) Boerema (White and Morgan-Jones 1987). Morgan-Jones and Burch (1987) elevated it to species rank, as *Phoma pinodella* (L.K. Jones) Morgan-Jones and Burch, because of significant and constant differences between it and *P. medicaginis,* but this terminology is rarely used in the literature.

Colonies of *P. medicaginis* var. *pinodella* on malt extract agar are dark brown to black with irregular patterns of pycnidia. Sometimes cultures will sector with abundant pycnidia

and light buff conidial exudate, reverse brown to black or with abundant crystals arranged in a fan shape (Punithalingham and Gibson 1976). Colonies are generally darker than *D. pinodes* and turn black at maturity, due to the abundant production of chlamydospores. Under a 12 h photoperiod of white light (wavelengths 350 and 750 nm), colonies form alternating zones of mycelium and pycnidia leading to concentric rings around the original mycelial plug. On V8 juice agar, in 12 h photoperiod, *P. medicaginis* var. *pinodella* grows faster than *D. pinodes* (Onfroy *et al.* 1999). As for *D. pinodes*, pycnidium production is increased with light and reduced at low temperature (Hare and Walker 1944). Pycnidia are sub-globose to variable in shape, and larger than those of *D. pinodes,* being 200 - 300 µm in diameter. Conidia are hyaline, smaller than those of *D. pinodes* at 4.5 - 9 x 2 - 3 µm, usually non-septate, occasionally 1-septate (Punithalingham and Gibson 1976). The rate of production of bicellular conidia on V8 juice agar (19 %) is significantly less than that of *D. pinodes* (Onfroy *et al.* 1999). Chlamydospores are dark brown, spherical to irregular, smooth to rough, terminal or intercalary and produced singly or in chains (Punithalingham and Gibson 1976).

The teleomorph of *P. medicaginis* var. *pinodella* has been reported only once, in culture, from material collected in Australia by S.M. Ali (Bowen *et al*. 1997) but it has not been described taxonomically as others have been unable to reproduce the teleomorph (Onfroy *et al*. 1999, Tivoli and Banniza 2007). Bowen *et al*. (1997) described the pseudothecia as larger than those of *D. pinodes*, being globose, dark brown, 140 - 250 µm wide and 170 - 420 µm high. Asci were reported to be cylindrical to sub-clavate 140 - 290 µm long and 20 - 30 µm wide, and ascospores hyaline, ellipsoid, guttulate, bicellular, constricted at the septum with rounded ends. They were similar in morphology to those of *D. pinodes* but larger, being 25 - 35 µm long and 12.5 - 19 µm at the widest point (Bowen *et al*. 1997).

P. medicaginis var. *pinodella* is found on *P. sativum* and other members of the Leguminosae family, and is isolated abundantly from the soil (Punithalingham and Gibson 1976). The role of *P. medicaginis* var. *pinodella* in ascochyta blight is considered secondary

to *D. pinodes* (Wallen 1964), though it has been isolated from all parts of the field pea plant (Hare and Walker 1944, Knappe and Hoppe 1995). It is particularly damaging when it causes foot-rot, especially in waterlogged soils (Hare and Walker 1944, Knappe and Hoppe 1995, Onfroy *et al*. 1999). This pathogen has also been recorded on clover pastures (*Trifolium* spp.) and lucerne and medic pastures *(Medicago* spp.), and isolates of *P. medicaginis* var. *pinodella* from these host species are capable of infecting field pea. The pathogen is reported to be particularly damaging on medics, causing defoliation and premature death (Barbetti and Khan 1987). *Trifolium* and *Medicago* pasture species are part of the Australian farming systems, and this has implications for the successful rotation of these crops and pastures.

1.3.3 *Ascochyta pisi*

A. pisi (Figure 3) is not known to produce a perfect state nor does it produce chlamydospores (Kraft *et al.* 1998b). Pycnidia are globose and brown, 100 - 200 µm in diameter (similar in size to *A. pinodes*) and conidiophores are hyaline and short, 6 - 14 x 3 - 8 µm. Conidia are hyaline, straight or slightly curved, 1-septate, slightly constricted at the septum with rounded ends, 10 - 16 x 3 - 4.5 µm. Colonies on oatmeal agar produce abundant pycnidia with carrot red spore exudate. *A. pisi* is found on *Pisum*, *Lathyrus* and *Vicia* spp. (Punithalingham and Gibson 1972a). *A. pisi* produces ascochitine, a metabolite toxic to *Pisum* species, which is not produced by either *D. pinodes* or *P. medicaginis* var. *pinodella* (Foremska *et al*. 1990, Marcinkowska *et al*. 1991).

1.3.4 Unidentified *Phoma* species

This fungus was isolated from commercial field pea seed in South Australia by Ali *et al*. (1982) and was initially mis-identified as *A. pisi* and later as *Macrophomina phaseolina* (Ali *et al*. 1982, Ali and Dennis 1992). Subsequent investigators classified this as an unidentified species of the *Phoma* genus (Dr. Hans de Gruyter, Plant Protection Services, Netherlands, personal communication 13th July 2006) and a detailed description of the species is required. A preliminary investigation, based on a few isolates, described the fungus as growing more

slowly on malt extract agar than *D. pinodes,* with pycnidia distributed in an irregular manner in the colony. Mycelium was sparse and light pink in colour. Conidia were hyaline, irregularly shaped but generally ellipsoid to ovoid 14 - 25 x 5 - 10 μm, non-septate or rarely 1-septate (Ali *et al*. 1982).

Symptoms on plants inoculated in glasshouse conditions comprised irregularly shaped brown to dark brown lesions, 0.5 - 2.0 mm in diameter. Longitudinal stem lesions coalesced to cover the stems and severe lesions occurred on stipules and leaves. Pycnidia appeared as scattered black dots on the stems within 7 days of inoculation, but very few developed on the leaves. Sclerotia developed on the stems 4 - 5 weeks after inoculation (Ali and Dennis 1992).

In glasshouse conditions, disease symptoms and pycnidia with viable spores can be produced on conventional cultivars of *P. sativum*, as well as a number of cultivars of *Medicago littoralis*, *M. scutella* and *Lens culinaris*. The latter three hosts exhibit small lesions, less than 1 mm in diameter, on the leaves and no stem lesions (Ali and Dennis 1992). It is important to establish the host range using modern cultivars of *P. sativum,* particularly the new semi-leafless types, and other crop cultivars grown in rotation with field pea that were not tested by Ali and Dennis (1992). The role of this pathogen in ascochyta blight epidemics in the field needs to be clarified.

1.3.5 Population structures and variation in pathogenicity

Individual isolates of *D. pinodes* vary widely in their ability to cause disease on field pea, ranging from producing a few necrotic flecks to causing large lesions, and variable reactions are sometimes noted between leaves and stems (Ali *et al.* 1978, Clulow *et al*. 1991a, Nasir and Hoppe 1991, Xue *et al.* 1998). It is not clear whether or not race specific interactions occur in the *D. pinodes* x *P. sativum* pathosystem (Tivoli *et al*. 2006) and, as a result, there is no method for classifying pathotypes of *D. pinodes*. Isolates have been designated pathotypes rather than races because the genetic basis of the resistance in the hosts has not been resolved (Onfroy *et al*. 1999). Wroth (1998b) found that the variation in mean disease scores for 99

isolates of *D. pinodes* on ten host genotypes was continuous. She concluded that the pathotype groupings designated by other researchers are based on arbitrary cut-off points within a continuous distribution. She showed the environmental component to be the major contributor to the overall variation in the host-pathogen interaction, with lesser contributions from isolate and genotype interactions. Onfroy *et al.* (1999) also reported no evidence of pathotypes among 50 French *D. pinodes* isolates.

There are significant differences in aggressiveness among isolates of *D. pinodes* but ranking of aggressiveness is similar across different genotypes of field pea (Wroth 1998b). Aggressiveness was not associated with geographic or host origin of the isolates and isolates from the same location and from the same crop are no more similar than those from widely dispersed sites or different host cultivars. However, Zhang *et al.* (2003) found that Canadian isolates were more virulent than those from Australia, New Zealand or European countries. *D. pinodes* populations are highly variable with respect to pathogenicity and it was recognised that changing seasonal conditions might alter the selection pressure on *D. pinodes,* potentially promoting a different proportion of the population (Wroth 1998b).

So far no physiological specialisation has been reported for *P. medicaginis* var. *pinodella* (Ali *et al.* (1978) though significant differences in aggressiveness among isolates have been observed (Onfroy *et al*. 1999).

A. pisi has a low level of population variability which appears to be stable. Five broad pathotype groups have been identified in the UK (Darby *et al*. 1986) and four races identified in Canada (Wallen 1957). Host reactions range from hypersensitive resistance to extreme susceptibility (Wallen and Jeun 1968).

Ali and Dennis (1992) reported that the unidentified *Phoma* sp. exhibited a high degree of pathogenic variability on conventional field pea cultivars and placed 15 isolates into 15 pathotypes. It is not known whether this variability is also exhibited on modern Australian field pea cultivars and whether it is linked to aggressiveness.

A number of serological and nucleic acid techniques have been used to facilitate the identification of the plant pathogens associated with ascochyta blight of field pea. The results from these studies suggest a closer relationship between *A. pinodes* and *P. medicaginis* var. *pinodella* than between the two organisms of the genus *Ascochyta* (*A. pisi* and *A. pinodes*).

Serological technique: A poly-clonal antiserum was prepared against soluble mycelial extracts of *D. pinodes* for enzyme-linked immuno-sorbent assay (ELISA). This test detected and quantified *D. pinodes* in infected seeds (Faris-Mokaiesh *et al*. 1995). In addition, two mono-clonal antibodies were produced which recognised either *A. pisi* or *D. pinodes* plus *P. medicaginis* var. *pinodella* but did not separate the latter two species (Bowen *et al*. 1996). No serological diagnostic research has been conducted on the unidentified *Phoma* sp.

Protein electrophoresis techniques: Isozyme and total protein electrophoresis was used to compare several *Ascochyta* and *Phoma* species. The soluble protein patterns of *P. medicaginis* var. *pinodella, P. medicaginis* var. *medicaginis* and *D. pinodes* were different from those of the *Ascochyta* spp., so isolates of *D. pinodes* and *Phoma* spp. were easily differentiated from *A. pisi*, but not from each other (Bouznad *et al*. 1997).

Nucleic acid techniques: Random amplified polymorphic DNA (RAPD) markers differentiated between *Ascochyta* species and distinguished among *A. pisi*, *D. pinodes* and *P. medicaginis* var. *pinodella* (Bouznad *et al*. 1995, Onfroy *et al*. 1999, Wang *et al*. 2000). RAPD analysis showed there is very limited intra-specific diversity among the isolates of the latter two species, though *D. pinodes* should have a higher degree of genetic diversity since it reproduces both sexually and asexually (Onfroy *et al*. 1999). While RAPD assays were able to distinguish between *D. pinodes* and *P. medicaginis* var. *pinodella*, the species could not be separated by Polymerase Chain Reaction - Restriction Fragment Length Polymorphisms (PCR-RFLP) in the rDNA regions. The internal transcribed spacers (ITS) of the fungi show no intra-specific and very little inter-specific variation (Fatehi *et al.* 2003, Peever *et al.* 2007), leading Fatehi *et al.* (2003) to conclude these were a single species. However, the inter-genic

spacer (IGS) region revealed that *A. pisi* differs from the other two species (Faris-Mokaiesh *et al*. 1996, Peever *et al.* 2007), and analysis of the protein coding gene glyceraldehyde-3-phosphate-dehydrogenase (G3PD) separated *D. pinodes* from *P. medicaginis* var. *pinodella* (Peever *et al. 2*007).

The highly conserved ITS region within these species has been used by the Root Disease Testing Service (RDTS) at the South Australian Research and Development Institute (SARDI) to develop a combined PCR detection test for *M. pinodes* plus *P. medicaginis* var. *pinodella*. Real-time PCR is used to quantify the pathogens present in soil or plant samples (Ophel-Keller *et al*. 2008).

Co-location of causal pathogens: The ascochyta blight pathogens are co-located on field pea plants (Bretag and Ramsey 2001) and also in soil (Wallen et al. 1967b). Le May *et al.* (2009) reported both antagonistic and synergistic interactions between *D. pinodes* and *P. medicaginis* var. *pinodella* when co-inoculated onto individual pea plants, depending on location and timing of application of conidia. There was a reduction in lesion size when the two pathogens were inoculated onto the same leaf, but synergistic or additive responses occurred when there was a 3 or 6 day period between inoculations on different leaves of the same plant (Le May *et al*. 2009). It is not known whether these pathogens directly compete for space and resources or whether they occupy slightly different niches that allow coexistence (Fitt *et al.* 2006). Investigations of any antagonistic or synergistic interactions between all the causal pathogens would increase our understanding of this fungal complex.

1.4 Epidemiology

1.4.1 Disease cycle

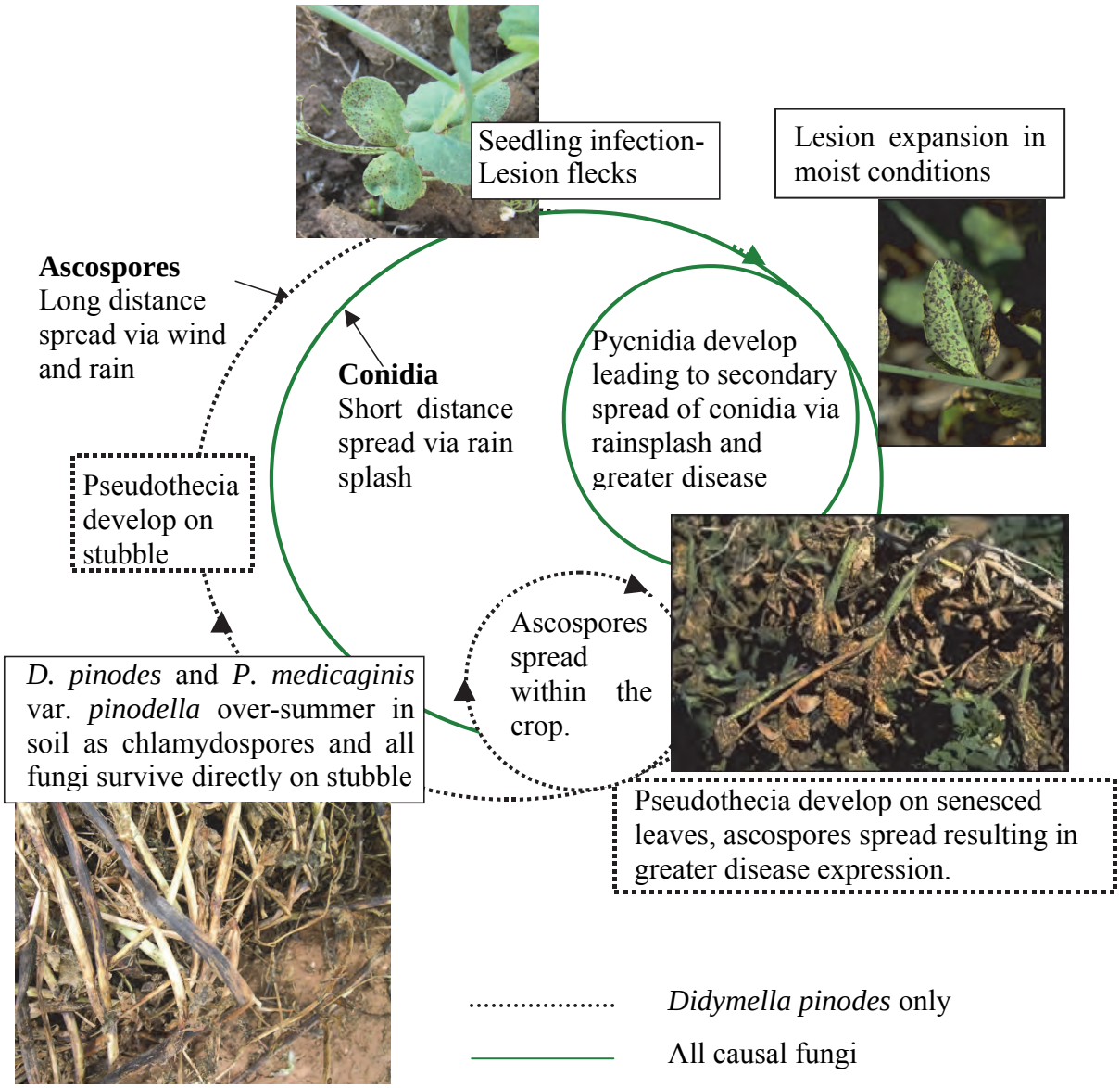
Figure 4 shows the disease cycle of ascochyta blight on field pea. Primary inoculum (asexual conidia or sexual ascospores) of all the causal pathogens is spread by wind and rain onto newly emerging crops (Carter and Moller 1961). As noted above, only *D. pinodes* produces ascospores (Punithalingham and Holliday 1972b), which develop from pseudothecia on

infested stubble or senescent plant material, whereas all causal pathogens produce conidia. Regardless of the type of inoculum, initial leaf infection results in small purple to black spots, which expand under moist conditions, killing the leaves (Roger and Tivoli 1996b). In France the initial small, flecked lesions are visible on plants with 10 - 20 nodes (Tivoli *et al*. 1996) while in Australia, ascochyta blight lesions may be visible on seedlings at the 4-node stage. Disease spreads rapidly, leading to leaf death. Pycnidia develop within the resulting lesions and conidia are spread to neighbouring plants (Schoeny *et al*. 2008). The conidial spread pattern leads to disease being more severe at the base of the plants than at median or upper parts of plants (Tivoli *et al*. 1996). In Australian conditions, conidia are considered of minor importance (Bretag *et al*. 2006) and ascospores are the primary factor in secondary spread of *D. pinodes*. These are produced within pseudothecia when infected leaves and stems become senescent, and are forcefully discharged into the air when moisture requirements are satisfied. The ascospores spread quickly throughout the crop and subsequent rainfall promotes rapid infection, increasing disease severity (Roger and Tivoli 1996b).

Stem infection may begin at the soil line and extends upwards, lesions often coalescing to girdle the stem. Stem girdling on seedlings can lead to seedling death (Hare and Walker 1944, Kraft *et al.* 1998b, Tivoli *et al.* 1996). When the fungi grow down the petiole of an infected leaf a stem lesion may begin at the base of the dead leaf, advancing above and below that point. Individual lesions eventually coalesce, girdling the stem (Hare and Walker 1944, Tivoli *et al*. 1996).

Infection of flowers causes them to wither and drop, while pod infection causes pods to become distorted and they may drop. This effect may be transient and is often unnoticed (Hare and Walker 1944). Young pods affected by disease become distorted. The pathogens survive between crops on stubble, on seed, or in soil (Wallen *et al.* 1967b).

Figure 4. Disease cycle of ascochyta blight of field pea caused by *Didymella pinodes*, *Phoma medicaginis* var. *pinodella*, and *Ascochyta pisi*.
(adapted from Tivoli and Banniza 2007)



1.4.2 Inoculum

1.4.2.1 Primary air-borne inoculum

Large numbers of ascospores, which are released from infested field pea stubble with the opening seasonal rains in autumn and early winter, have been identified as the primary source of inoculum for *D. pinodes* in Australia (Bretag 1991, Carter and Moller 1961, Carter 1963). These spores become wind-borne and travel at least 2 km (Bretag and Ramsey 2001). The asci do not mature all at once and a perithecium may discharge spores several times (Hare and Walker 1944). Successive crops of mature pseudothecia develop on field pea residues, initially in very large numbers, exhausting the inoculum by 50 weeks (Carter 1963, McDonald and Peck 2009, Peck *et al*. 2001). In France there are fewer airborne ascospores at the beginning of the growing season than in Australia and disease is not severe until spring when the crop begins to mature (Schoeny *et al. 2*007).

Moisture is an important factor in ascospore release. When pseudothecia become moist the asci enlarge and rupture at the tip, releasing all of the ascospores into the air at once (Carter 1963, Hare and Walker 1944). Analyses of hourly ascospore counts showed that dew is effective in causing release of ascospores but that the largest numbers occur in periods of rainfall (Carter and Moller 1961, Carter 1963). In controlled experiments, 4 - 6 h of wetness at 15 - 20 ºC was required for ascospore release, whereas longer times were required at lower temperatures. A diurnal pattern of spore release was noted, with the peak in late afternoon and the lowest point in the middle of the night, possibly as a response to the photoperiod (Bretag and Lindbeck 2006, Carter 1963). No spores are released if the pseudothecia are kept dry, and temperature has no obvious effect on release of spores (Hare and Walker 1944).

On plant tissue, abundant pseudothecia develop on dead parts of growing plants, at any time of the season on tendrils, petioles or leaves and also on stubble. They will generally develop 3 - 4 weeks after senescence, but this varies depending on availability of moisture. When in continuous moisture, the pseudothecia may develop within 12 days of inoculation.

Temperatures between 16 ºC to 28 ºC have little effect on development of pseudothecia on plant material although, as stated above, the numbers decrease at temperatures above 20 ºC in pure culture (Hare and Walker 1944).

1.4.2.2 Soil-borne inoculum

The pathogens, except *A. pisi*, survive in the soil for a number of years and grain yields have been strongly correlated with the amount of soil-borne ascochyta blight fungi (Bretag and Ward 2001). This source of inoculum is particularly important in established field pea growing areas where soil-borne inoculum builds up under successive field pea crops (Wallen and Jeun 1968). *P. medicaginis* var. *pinodella* has been isolated from soil up to 5 years after field pea was grown and *D. pinodes* from soil not sown to field pea for 20 years (McDonald and Peck 2009, Peck *et al*. 2001, Wallen *et al*. 1967b, Wallen and Jeun 1968). The pathogens may cause foot-rot by infecting the base of the stem and the epicotyl, or inoculum may be rain splashed from the soil onto the leaves of new crops (Clulow *et al*. 1991b, Sakar *et al*. 1982).

The pathogens survive as chlamydospores, mycelium or sclerotia. The former can withstand temperatures of at least 100 ºC for 12 - 15 h (Wallen *et al*. 1967b) and survive for more than 12 months in inoculated sterile soil maintained at –20 ºC - 25 ºC. All the chlamydospores incubated below 15 ºC remained infectious. None of the pathogens survived for more than 1 month at 35 ºC or 4 months at 30 ºC (Wallen and Jeun 1968).

D. pinodes is also a moderate saprophyte (Dickinson and Sheridan 1968, Sheridan 1973), demonstrated by an increase in detectable levels in soil in the 6 - 12 months following harvest, after an initial decline in the first 6 months. Burying infested stubble decreased the survival time to below 12 months (Davidson *et al*. 1999, Sheridan 1973, Zhang *et al*. 2005) possibly by preventing saprophytic growth through depletion of oxygen and microbial activity.

Whereas *A. pisi* has a very limited ability to survive or grow in the soil and has not been shown to produce chlamydospores, it can survive in sterile soil for 12 months at cold

temperatures, such as – 20 ºC - 5 ºC. However, its survival is reduced at 15 ºC and it dies out quickly at 30 ºC - 35 ºC (Dickinson and Sheridan 1968, Wallen and Jeun 1968, Wallen *et al.* 1967b). *D. pinodes* and *P. medicaginis* var. *pinodella* both are strongly antagonistic to *A. pisi* in the soil, further reducing the population of this pathogen (Wallen and Jeun 1968).

There is no information on whether the unidentified *Phoma* species can survive in soil or whether soil-borne inoculum of this pathogen is associated with ascochyta blight disease.

1.4.2.3 Seed-borne inoculum

All the pathogens can infect seed (Kraft *et al*. 1998b, Maude 1966). In Australian seed lots, up to 90 % of samples may be infected, most frequently with *D. pinodes* but also with significant incidence of the unidentified *Phoma* species or *P. medicaginis* var. *pinodella (*Ali *et al.* 1982, Bretag *et al.* 1995b). *A. pisi* has been detected at a very low level (1 %) in only one (Bretag *et al.* 1995b) of three studies (Ali *et al.* 1982, Bathgate *et al*. 1989). Surface sterilisation of the seed resulted in a decrease in the recovery *of D. pinodes* from 60 % to 18 %, leading to the conclusion that the majority of this pathogen is carried on the seed coat (Bathgate *et al*. 1989). Deep-seated infection is more common in the seed lots with highest rates of infection (Bretag *et al.* 1995b). Incidence of seed infection dropped rapidly in the first 2 years of storage, followed by a gentler decline in the following years (Bretag *et al*. 1995b).

Seed infection is increased by several factors *viz*. spring rainfall which disperses inoculum, early sown crops which have severe disease due to exposure to large numbers of airborne ascospores, and late harvested crops that are more likely to be exposed to secondary inoculum (Bretag *et al.* 1995b). Seed lots from low rainfall areas i.e. less than 350 mm per annum, may be free of the pathogens, making these areas suitable for producing pathogen-free seed (Bathgate *et al*. 1989).

In controlled conditions, seed to seedling transmission to the basal plant parts under the soil is frequent, eg. 40 % for *A. pisi* and 100 % for *D. pinodes*, leading to death of young seedlings (Maude 1966, Xue 2000). However the disease does not spread to the higher parts

of the plant suggesting that seed infection is not an important source of inoculum for an ascochyta blight epidemic (Bretag *et al*. 1995b, Moussart *et al*. 1998). Consequently, higher seeding rates may compensate for losses incurred through seed infection (Bretag *et al*. 1995b) when *D. pinodes* or *P. medicaginis* var. *pinodella* are present. When more than 11 % of seed is infected, serious losses can occur in the field through poor emergence (Bretag *et al.* 1995b, Moussart *et al*. 1998, Xue 2000), and low temperatures during early crop development can increase such losses. While *P. medicaginis* var. *pinodella* may induce severe stem and crown lesions at the seedling stage, these lesions do not expand as fast as those due to *D. pinodes* (Knappe and Hoppe 1995). The role of seed-borne infection of the unidentified *Phoma* sp. in an ascochyta blight epidemic has not been identified.

1.4.2.4 Secondary inoculum

During crop growth, both pycnidia and pseudothecia have been observed on the same plant organs, but whereas pycnidia are produced on both green and senescent plant organs, pseudothecia only appear on senescent parts, first appearing just before flowering. Discharge of both types of spore is initiated by rainfall or dew so that epidemics are more severe in wetter conditions (Roger and Tivoli 1996b). The majority of conidia are splashed downwards with few remaining at the infectious node and very few moving upward (Schoeny *et al.* 2008). When ascospores are produced, disease spreads rapidly to the top of the plant canopy. The greatest damage due to the disease is caused by this secondary spread of ascospores (Bretag 1991, Hare and Walker 1944).

Pseudothecia of *D. pinodes* may appear 18 days after lesions first appear. Since they mostly form on senescent stems, the oldest organs bear the greatest number. Ascospores dispersal is via wind, resulting in uniform infection over the field. Spore dispersal is diminished as the canopy develops and acts as a barrier to ascospore dispersal. The numbers of ascospores usually remain comparatively low until the end of the season when pseudothecia increase on senescent material, and then ascospore counts may treble (Roger and

Tivoli 1996b). Large numbers of airborne spores of *D. pinodes* as secondary inoculum have been associated with spring rains in Australian conditions but the numbers vary widely depending on seasonal conditions (Davidson *et al.* 2006, McDonald and Peck 2009, Peck *et al.* 2001). A potentially important part of disease control is to delay senescence and the development of pseudothecia past flowering by decreasing the rate of disease progress on the plant (Roger and Tivoli 1996b).

Pycnidia may form within 11 days after the appearance of symptoms and increase in number until the end of the crop vegetative cycle. Roger and Tivoli (1996b) found they formed when coalesced lesions covered approximately 25 % of the leaf area (1996b). In France, the numbers of conidia are similar to those of ascospores throughout the season, but without the final rapid increase, and conidia spread only short distances, 20 cm above the soil surface (Roger and Tivoli 1996b, Schoeny *et al. 2*007).

Ascospores are much more efficient than conidia at disseminating the fungus, as shown by Hare and Walker (1944). Large numbers of ascospores were captured on slides in infected fields during rainy periods, up to 129 ascospores cm^{-2} within the plot and 8 ascospores cm^{-2} at 305 m from the infected site. No conidia were captured on the slides even though they were present on the field pea plants. Few researchers have studied secondary inoculum in Australian conditions. Bretag and Lindbeck (2006) did not discover any pycnidia of *D. pinodes* on field pea at the one site investigated (Horsham, Victoria) but this finding needs to be confirmed.

1.4.3 Infection process

Conidia of *D. pinodes* germinate with one or more germ tubes, which frequently branch and form appressorium-like structures on the leaf and cotyledon surface 6 h after inoculation (Clulow *et. al.* 1991b, Roger *et al*. 1999a). Penetration occurs through the epidermal walls 8 h after inoculation (Nasir *et al*. 1992, Roger *et al*. 1999a), not via the stomata (Hare and Walker 1944), and an infection vesicle is formed, lying partly in the epidermal wall and partly in the

cell lumen. From this, a penetration hypha arises and initiates intra- and inter- cellular hyphae (Nasir *et al*. 1992). Penetration occurs within 24 h of inoculation, with the aid of cell-wall degrading enzymes (Heath and Wood 1969, Roger *et al*. 1999a) and the rapid colonisation by *D. pinodes* is soon followed by tissue collapse and browning of the invaded cells (Heath and Wood 1969) in both resistant and susceptible genotypes (Nasir *et al. 1*992). In resistant types, the formation of infection vesicles and penetration hyphae is reduced and the development and spread of lesions is retarded (Nasir *et al*. 1992). Symptoms may appear within 24 h of inoculation, and consist of brown spots, 2 mm in diameter, which continue to grow and coalesce, killing the entire leaf, and pycnidia can form in 3 days (Heath and Wood 1969, Roger *et al*. 1999a). Symptoms on the cotyledon may not be visible until 4 days after inoculation (Clulow *et al.* 1991b). With continuous wetness, stem lesions can girdle the stem in 9 days (Clulow *et al.* 1991b). Increasing inoculum concentration increases disease severity by escalating the rate of lesion expansion (Heath and Wood 1969, Roger *et al*. 1999a) but, inexplicably, does not affect the lesion number (Heath and Wood 1969). The literature indicates there has been no clarification of this aspect since the study was conducted.

D. pinodes develops faster than *A. pisi* within field pea leaves. While conidia of both fungi germinate at a similar rate on leaf surfaces, the percentage of spores that penetrate the host is 26 % higher for *D. pinodes* than *A. pisi*. In addition, lesions due to *D. pinodes* expand 10 times faster than those due to *A. pisi* and the lesions are 3 - 6 times larger in diameter (Heath and Wood 1969). This difference may be due to the production of the phytoalexin, pisatin, by the plant. Pisatin can be detected in host tissue 24 h after field pea leaflets have been inoculated with *D. pinodes* or *A. pisi.* It reduces germ-tube growth of both pathogens, and limits the expansion of lesions due to *A. pisi*. Pisatin can be degraded to non-toxic forms by pathogenic strains of *A. pisi* and, under conditions of leaf wetness or high humidity, by *D. pinodes*. The presence of water ensures there is a ready supply of nutrients in solution, allowing *D. pinodes* to break down the pisatin. Pisatin restricts lesions due to *D. pinodes* only

under dehydrated conditions, when nutrient supply is restricted, allowing a large accumulation of pisatin (Heath and Wood 1971).

1.4.4 Effect of temperature, moisture and wind on infection and disease

Conidia of *A. pisi*, *D. pinodes* and *P. medicaginis* var. *pinodella* germinate at similar temperatures, 4 - 24 ºC, with an optimum temperature of 28 ºC in distilled water (Hare and Walker 1944). The optimum temperature for the infection process and expansion of lesions due to *D. pinodes* is 20 ºC, at which germination begins 2 h after inoculation, with 70 % of conidia germinated after 72 h (Roger *et al*. 1999a). Disease severity and pycnidial numbers are greatest at 20 ºC, leading to visible symptoms 24 h post-inoculation and pycnidia after 3 days. Longer wetness periods are necessary at sub-optimal temperatures and at 5 ºC the incubation period, i.e. from inoculation to symptom development, is 7 days (Roger *et al*. 1999a).

Roger *et al*. (1999a) converted latent periods, i.e. from inoculation to pycnidial development, of *D. pinodes* to degree-days and found they were similar for 5 ºC, 10 ºC, 15 ºC and 20 ºC under continuous leaf wetness. The plants developed one leaf in 58 degree-days and the fungus went through one cycle from inoculation to fruiting body formation in the same period, hence the pathogen developed at the same rate as new leaves were produced. In contrast, under conditions of high humidity (95 – 100 %) rather than free water on the leaf, the pathogen exhibited an extended latent period and pathogen growth lagged behind the plant growth, except at 20 ºC where no increase in latent period was observed. These results may explain why in periods of leaf wetness in winter and early spring the disease keeps pace with plant growth but in spring, when periods of leaf wetness may be infrequent, plant growth may exceed that of the pathogen (Roger *et al*. 1999a). Frequent rain in spring can lead to conditions of leaf wetness and optimum temperatures that could allow rapid expansion of the epidemic. The latent period was also affected by growth stage i.e. there was a longer latent period if plants were inoculated at the final stage of seed abortion, when seed length is 6 mm,

than 15 days later (Roger and Tivoli 1996b). This may be because older, lower leaves of the canopy are more susceptible since pisatin production decreases as leaves senesce (Bailey 1969 cited in Tivoli *et al*. 1996). The effect of cultivars with differing plant architectures or growth patterns was not studied but this may also influence the relationship between pathogen cycles and plant growth.

Disease severity was not affected by interruption in wetness immediately after inoculation, prior to spore germination. Thus the ungerminated conidia can survive on the leaf surface for dry periods of up to 21 days. Disease development is interrupted if the dry period occurs during germination and the longer the dry period, the lower the subsequent spore viability. If a dry period occurs during appressorium formation, symptoms appear later and disease remains slight, though limited appressorium formation and hyphal penetration may continue through the dry period. Resumption of appressorium formation and hyphal penetration is infrequent (<5 %) when leaves are re-wetted. After the fungus has penetrated the leaf, a dry period has no impact on disease expression (Roger *et al*. 1999b). In Australia intermittent dry periods are not uncommon during the growing season and studies similar to those described above may identify combinations of cultivars with differing architecture x regions most likely to avoid severe disease.

The effect of the temperature and moisture upon disease onset has been modelled using daily weather data, assuming that disease onset occurs once the temperature and moisture requirements for infection are met (Schoeny *et al*. 2007). This model is aimed to develop a rational fungicide application strategy in France by timing the spray applications to coincide with predicted infection periods. Foliar fungicides in Australian field pea crops are often uneconomic and the industry is unlikely to adopt a strategy that involves numerous applications within a single crop.

Temporal and spatial dynamics of ascochyta blight were studied in field epidemics from an inoculum source of 36 m^2 in Canada (Zhang *et al*. 2004). The slope of the disease progress

curve increased with distance from the inoculum source and epidemic onset was delayed with increasing distance from the inoculum source. Disease gradients were closely related to wind direction, spreading further downwind, but this can vary according to conditions. A disease spread model was developed which combined direction, distance and time, to describe disease in time and space. A point source of inoculum usually produced a steeper gradient than an area source (Zhang *et al.* 2004). While the 36 m^2 source inoculum used in this study was quite large, it is still much smaller than the inoculum sources likely to occur Australian conditions i.e. an entire field of field pea stubble of at least 100 ha. Regional scale spread of ascochyta blight has been predicted in Western Australia in an area 30.9 x 36.8 km to determine disease risk in field pea crops and the model still requires large scale validation in other areas (Salam *et al.* 2011a). It was reported that when field pea cropping covers 3.7 % of the area, the ascospores can potentially spread across four-fifths of the region. This information assists farmers to select fields for field pea crops that are at a low risk of infection from ascospores. However, this density of field pea crops is lower than in South Australia, where it is more than 6 % (McMurray and Seymour 2005), and the dispersal model needs to be validated in this higher cropping density.

1.5 Effect of ascochyta blight on yield of field pea

The percentage yield loss due to ascochyta blight in field pea has been correlated with Area Under the Disease Progress Curve (AUDPC), as well as with disease scores on stipules and stems shortly before harvest (Su *et al*. 2006, Tivoli *et al*. 1996). Bretag *et al*. (1995a) calculated that in the field there is a 5 - 6 % loss in yield for every 10 % of stem area affected, with greatest losses in early maturing cultivars. The disease may affect yield indirectly by reducing biomass (Garry *et al*. 1998b, Tivoli *et al.* 1996) or directly through pod infection (Beasse *et al*. 1999).

Ascochyta blight decreases the photosynthetic capacity of leaves of the field pea plant (Beasse *et al*. 2000, Garry *et al*. 1998a), mainly through reduced radiation use efficiency

(RUE). The RUE in the diseased plants was 30 - 60 % that of healthy plants during seed fill and 50 - 70 % that of healthy plants at physiological maturity. Earlier, at the beginning of seed fill, there was no difference in the RUE of diseased and healthy plants (Beasse *et al.* 2000, Le May *et al*. 2005). There was only a slight decrease in radiation interception efficiency (RIE) throughout the epidemic. The small effect on RIE was probably because the epidemics become serious in spring when the canopy was fully formed and the disease decreased RIE only by accelerating senescence of the leaves (Beasse *et al.* 2000).

Crop growth in diseased field pea crops was modelled (Beasse *et al*. 2000) for six spring field pea cultivars with different architecture (Le May *et al*. 2005). A model was based on the combination of disease progress in the canopy (i.e. number of nodes affected by disease) and the canopy structure (leaf area index). Photosynthesis in all six cultivars was equally affected by amount of disease. If disease severity was at least 10 - 15 % leaf area diseased on node 13 and below, then plant growth was generally reduced after the beginning of seed fill, when leaf area was no longer expanding (Beasse *et al*. 2000). However, differences in canopy structure affected disease progress up the plant so the reduction in plant growth and yield was variable between cultivars. The most serious effect on growth and yield occurred in cultivars where the canopy was short and the disease progress rapid. There was a slight deviation from the predicted disease progress for those cultivars that senesced earlier than others (Le May *et al*. 2005).

Nodes infected during flowering have reduced seed number per pod, reduced mean seed weight (Garry *et al*. 1996, Garry *et al.* 1998b, Tivoli *et al*. 1996, Xue *et al*. 1997) and reduced pod number (Wallen 1974, Xue *et al*. 1997). Infection before the beginning of seed fill reduces seed number but not mean seed weight since a reduction in seed number from early disease onset may result in more assimilates being allocated to the remaining seeds (Garry *et al*. 1998b). Because of the effect on seed number, there is a larger effect on yield if inoculation occurs at flowering rather than at seed fill even if the eventual disease score at the

end of the season is the same (Garry *et al.* 1998b). Xue *et al.* (1977) calculated yield loss of 24 - 34 % associated with early epidemics compared with 19 % loss if inoculation occurred at seed fill stage. Infection after the beginning of seed fill does not reduce seed number but does reduce seed weight by as much as 40 % (Garry *et al.* 1998b). There is a linear relationship between disease severity on pods and decrease in seed weight, to a maximum of 20 %. This is consistent with the contribution of the hull to seed nutrition (Beasse *et al*. 1999). Severe disease affects the carbohydrate/nitrogen ratio so that seed protein concentration increases and starch concentration decreases (Garry *et al*. 1996).

In France, infection at seedling stage (6 - 7 leaf) resulted in no more loss than when infection occurred at the beginning of flowering. This may be due to the late expansion of ascochyta blight epidemics in European conditions where a low level epidemic until flowering is common for spring-sown field pea (Beasse *et al. 2*000). In epidemics that develop earlier, which may happen in Australian winter-sown field pea, the effect of early disease on yield has not been investigated. The model developed in France (Beasse *et al*. 2000, Le May *et al*. 2005) implies that the effect of the disease on plant growth and yield is solely a result of reduced photosynthesis, with no compensatory effect by healthy leaves. They concluded that, in European conditions, stem girdling due to ascochyta blight does not affect growth unless the stem is girdled before flowering. However, in Australia, the environment is much drier during crop maturation than Europe, and the effect of stem girdling on yield in this environment needs to be understood. Similar modelling in Australian conditions would assist in developing economic disease control methods, particularly strategic foliar fungicide applications.

1.6 Disease control

1.6.1 Agronomic practices

Rotation intervals of more than 5 years between field pea crops and delayed sowing are the main recommendations given for ascochyta blight control in Australia. Shorter rotation intervals are associated with severe ascochyta blight and lower yields (Davidson and Ramsey 2000, Peck and McDonald 2001), and the wider rotations were recommended early in the history of field pea farming in South Australia (McAuliffe and Webber 1962), due to the survival of soil-borne inoculum for at least 10 years (Wallen *et al*. 1967b, Wallen and Jeun 1968, Sheridan 1973).

In Australia, early sowing dates are correlated with increased severity of ascochyta blight (Davidson and Ramsey 2000, Peck and McDonald 2001), owing to the release of airborne ascospores from field pea stubble during rain in autumn and early winter (Carter and Moller 1961, Carter 1963, Bretag *et al.* 2000, Davidson *et al*. 2006). In South Australia, ascospores numbers are known to reach a peak and then decline rapidly after 70 - 80 mm of autumn and winter rainfall (autumn beginning 1st April). It has been suggested that, in seasons when autumn rainfall begins earlier than usual, field pea crops could be planted early, i.e. in May, and still avoid severe ascochyta blight (Peck *et al. 2*001, McDonald and Peck 2009). In France, however, later sowing dates are correlated with later onset of disease (Schoeny *et al. 2*007). Early sowing in Australia has the added effect of increasing incidence of infection in harvested seed (Bretag *et al. 2*000). Reduction of disease by delayed sowing may result in a yield increase of 0 - 40 %, depending on seasonal conditions (Bretag *et al*. 2000). Late maturing cultivars have lower disease scores than early maturing cultivars, possibly due to the later development of necrotic tissue and hence later development of pseudothecia (Tivoli *et al.* 1996). However, late maturing types are generally unsuited to Australian growing conditions due to limited soil moisture during crop maturation (Bretag *et al*. 2000).

The effect of temperature and moisture on pseudothecium development and ascospore maturity has been modelled to predict the timing of ascospore release for different regions and seasons (Salam *et al.* 2011b). Validation using 16 Western Australian field pea trials identified that spore loads of less than 40 % at sowing were related to low disease severity (Salam *et al.* 2011c). Growers use this model ('G1 Blackspot Manager') to determine optimum sowing dates to avoid the peak of ascospore release, with the recommendation that the ascochyta blight risk from airborne spores is minimised once more than 60 % of the spores have been released. The model is used in conjunction with other agronomic disease control measures, such as appropriate rotation interval between field pea crops to minimise soil-borne inoculum and distance from infested stubble. The applicability of these recommendations to different agro-ecological zones has not been determined, nor has the model been assessed for the absolute numbers of spores produced. 'G1 Blackspot Manager' predicts the fraction of ascospores available in a given season but the absolute numbers may vary widely with the season, proximity to infested stubble and the severity of disease on the stubble. The effect of amount of ascospores available as inoculum on disease severity is not understood.

Pea stubble can be ploughed into the soil immediately after harvest, before the ascospores can be dispersed by wind and rain (Kraft *et al.* 1998b). Canadian studies have shown that tilling and burying infested field pea stubble can lead to substantial reduction in ascochyta blight severity on subsequent field pea crops, early in the growing season. However, the difference in disease severity is minimal by the end of the season (Bailey *et al.* 2001). In light and dry soils such as occur in Australia, ploughing stubble into the soil is not recommended, as it may result in soil erosion during summer.

Sowing density has no effect upon disease on pods but has been shown to increase the percentage of stems encircled by lesions (Tivoli *et al.* 1996). Seeding rates generally have little effect on disease severity (Beasse *et al*. 2000, Bretag *et. al* 1995, Tivoli *et al*. 1996),

probably because splash dispersed conidia are likely to re-infect the same plant on which they developed (Schoeny *et al.* 2008).

Ascochyta blight is more severe in crops with poor nutrition or other adverse environmental factors. Growing field pea in water-logged soils increased severity of infection by *D. pinodes* and reduced plant growth. Cultivars with greater sensitivity to water-logging suffered higher yield losses from the disease (McDonald and Dean 1996). Mechanical injury to plants, through sandblasting, increased disease severity on plants that become infected within 48 h of the injury. However, later in the season disease intensity showed no apparent differences indicating that injury plays a minor role in the establishment and development of *D. pinodes* (Banniza and Vandenberg 2003). Severe crop losses attributed to the unidentified *Phoma* species have been reported in field pea following post–emergence herbicide sprays, which place additional stress on the crop (Ali and Dennis 1992). The herbicides diuron, metribuzin and fluazifop have been reported to increase ascochyta blight crown lesions caused by all of the pathogens, while foliar levels of the micronutrient manganese were negatively correlated with severity of crown lesions (Davidson and Ramsey 2000).

1.6.2 Seed treatments and foliar fungicides

Seed treatment with fungicides, such as captan or thiram, can reduce the transmission of seed-borne ascochyta blight, though in field trials captan has been shown to reduce emergence (Ali *et al*. 1982, Kraft *et al*. 1998b, Maude 1966, Wallen *et al*. 1967a, Xue 2000). However, very few Australian growers use seed dressings to control ascochyta blight in field pea due to the perceived lack of economic benefit from this practice (Peck and McDonald 2001).

Very few South Australian field pea growers use foliar fungicides to control ascochyta blight in field pea (Peck and McDonald 2001) since low commodity price and high cost of the fungicides generally make this practice uneconomic (Bretag 1991). Similarly, in Canada, disease may be reduced by foliar fungicide applications during flowering (Wallen 1964, Warkentin *et al*. 1996, Warkentin *et al*. 2000) but applications are not cost effective. This is

particularly so in seasons with low disease pressure and low yield potential, meaning that seasonal rainfall needs to be considered before applying fungicide (Xue *et al*. 2003). In France, field pea crops are often sprayed with protectant fungicide at 10 - 15 day intervals from flowering through to harvest (Beasse *et al*. 1999, Schoeny *et al.* 2007). Chlorothalonil, mancozeb, prochloraz and procymidone can reduce disease and increase seed weight by up to 5 % and yield from 3 to 33 %, as well as reduce infection on harvested seed (Nasir and Hoppe 1997, Thomas *et al*. 1989, Warkentin *et al*. 1996, Warkentin *et al*. 2000). There is recent evidence that foliar fungicides provide economic benefit in Australia when applied to the cultivars Kaspa and Parafield, provided that the crop yield is at least 2.5 t ha^{-1} (Davidson *et al*. 2004).

The strategic use of foliar fungicides to control ascochyta blight in Australian field pea crops requires further investigation. As mentioned above, delaying the secondary production of pseudothecia may lead to effective disease control. This means avoiding premature senescence through low planting densities and good crop management, and applying fungicides before senescence to delay the rate of disease progress (Roger *et al*. 1999a). Protection from ascospores at flowering would also prevent or reduce pod infection and flower abortion. The identification of the time when pycnidium formation begins might also be used to initiate fungicide sprays (Roger and Tivoli 1996b). Protectant fungicides need to be applied before infection occurs and predictions of wet and dry periods using risk models may assist in timing these fungicides (Roger *et al.* 1999a).

1.6.3 Host resistance

1.6.3.1 Inheritance of resistance

The inheritance of resistance to ascochyta blight in field pea is complex and poorly understood. There are at least three separate pathogens, resistance to each is under different genetic control, and for each pathogen, the resistances to foliar and root infections also appear to be under different genetic control (Sakar *et al*. 1982).

No single gene or major gene resistance to *D. pinodes* has been found despite extensive searches. Resistance is quantitative, small but heritable, and the environment, including leaf age, inoculum concentration and temperature, affects the expression of resistance (Clulow 1989, Wroth 1999, Zhang *et al*. 2006, Zimmer and Sabourin 1986). Minor genes, identified in a number of studies (Ali-Khan *et al.* 1973, Ali *et al*. 1978, Ali *et al*. 1994, Prioul *et al*. 2003, Xue and Warkentin 2001), need to be combined to develop resistance to *D. pinodes* (Kraft *et al.* 1998a, Wark 1950, Zhang *et al*. 2006) but it may be necessary to widen the genetic base and introgress genes from wild genotypes (Clulow *et al.* 1991a, Fondevilla *et al. 2*007, Gurung *et al. 2*002, Wroth 1998a, 1999, Zimmer and Sabourin 1986). Some attempt has been made to identify molecular markers for disease resistance and 13 quantitative trait loci (QTL) for ascochyta blight resistance have been identified on seven linkage groups (Timmerman-Vaughan *et al. 2*002). Unfortunately, progress in breeding for resistance has been slow and, while cultivars with high susceptibility are no longer grown commercially, no highly resistant germplasm has been detected (Bretag *et al.* 2006).

It has been reported that a dominant gene controls resistance to *P. medicaginis* var. *pinodella* (Rastogi and Saini 1984). Some resistance to *P. medicaginis* var. *pinodella* foot-rot symptoms is due to the phenolic substances contained in the pigmented testa but other lines with a non-pigmented testa also show good resistance (Knappe and Hoppe 1995, Sakar *et al*. 1982). Foliar screening for response to this pathogen shows that most lines are susceptible to very susceptible, with severity increasing with plant age. There appears to be no correlation between foot-rot and foliar disease score (Nasir and Hoppe 1991, Sakar *et al*. 1982) though symptoms were significantly correlated in one study (Hillstrand and Auld 1982). There is no correlation between foot-rot and foliar symptoms for *D. pinodes* (Ali *et al*. 1978, Xue *et al*. 1996).

Resistance to *A. pisi* is durable and determined by three dominant genes and two complementary genes, as well as further single dominant and recessive genes (Darby *et al*.

1985, Lyall and Wallen 1958, Wallen *et al*. 1967a, Wark 1950). However, pathogenic variability determines that before resistant cultivars are grown in a region, they must be confirmed as resistant to the local pathotypes of the fungus (Bretag and Ramsey 2001, Darby *et al*. 1986, Wallen 1957).

Ali *et al*. (1978) identified potential sources of moderate resistance to the unidentified *Phoma* species, though no single source was effective against all pathotypes and one isolate was pathogenic on all 58 field pea lines tested. Further research is required to identify effective resistance to this pathogen.

1.6.3.2 Resistance in plant organs

Disease progresses more rapidly on stipules and leaves than on stems, so leaf or stipule resistance may be important to slow the rate of epidemic development (Roger and Tivoli 1996b). However, stem lesions are thought to cause the main yield loss (Wallen 1974), so breeders should target resistance to infection in the stem. Leaf and stem resistances are thought to be controlled by different genes (Clulow *et al*. 1992). On stems, resistance to *D. pinodes* is expressed mainly in the pre-penetration phase, leading to the failure of appressoria to develop. Any attempted penetration results in a hypersensitive response in the underlying cells (Clulow *et al.* 1991b, 1992) but this does not provide reliable resistance as extended moisture periods of 40 h or higher inoculum concentrations can overcome this barrier (Wroth 1998b). *D. pinodes* penetrates the cuticle of leaves of both resistant and susceptible lines, and resistance is expressed only after penetration (Clulow *et al.* 1992). In resistant plants, cell death is delayed and limited to a few cells around the hyphae, and, 10 days after inoculation, fungal colonisation is limited to about 1 mm within the site of penetration. However, the fungus can be re-isolated from the point of inoculation, indicating that the resistant hosts do not kill the pathogen. By comparison, necrotic lesions in susceptible lines spread more than 10 mm in diameter in the same period, and hyphae can be re-isolated from green leaf tissue beyond the necrotic lesion. Cell death occurs in advance of the colonised area by 2 to 3 cells

(Clulow *et al.* 1992) through the action of cell-wall degrading enzymes which are known to be produced by *D. pinodes*, *P. medicaginis* var. *pinodella* and *A. pisi* (Heath and Wood 1969, 1971). It is not known whether the unidentified *Phoma* sp. produces similar enzymes or any toxic metabolites. While stem and leaf resistance seem to be inherited independently (Clulow *et al.* 1991a, Xue and Warkentin 2001), pod infection (% pod area diseased) has been positively correlated with both seed and leaf infection (Xue and Warkentin 2001).

Breeding for stem strength may be an alternative method of reducing severity of losses due to ascochyta blight. Weak stems result in lodging and humid crop canopies, leading to an increase in ascochyta blight (Banniza *et al.* 2005). Ascochyta blight resistance and stem strength are independent traits and concurrent genetic gains should be feasible within a breeding programme (Beeck *et al*. 2006). Lodging is negatively correlated with the proportion of lignin, cellulose, fibre and xylem in the stem sections. Most fungi are not able to degrade lignin and this may be an important component of stem resistance (Banniza *et al*. 2005). The importance of stem strength and prevention of lodging in reducing the severity of ascochyta blight epidemics needs further investigation.

In addition, cultivars with long internodes had less severe disease on stipules and reproductive nodes than cultivars with shorter internodes (Le May *et al.* 2008). It was hypothesised that this is because splash dispersal is more effective on cultivars with short internodes, while splash dispersal may fail on those with long internodes. Le May *et al*. (2008) concluded that the optimum canopy architecture of field pea to reduce ascochyta blight included long internodes and small leaf area index to reduce leaf wetness duration.

1.7 Methods for disease assessment

Disease progress factors in the field such as disease severity, AUDPC and rates of disease increase are considered to be the most useful means of discriminating between field pea genotypes in terms of their response to ascochyta blight. The disease progress curves on 14 European cultivars all had a similar shape, although the cultivars exhibited differences in

disease severity (Prioul *et al*. 2003). Field studies have shown that the greatest disease severity occurs earlier for some cultivars than others and towards the height of an epidemic there is little difference in rankings of genotypes (Wroth and Khan 1999). Data from a single assessment date are likely to be biased towards those cultivars that express disease later (Bretag 1991) and so successful resistance screening requires multiple disease assessments over time.

At the beginning of an ascochyta blight epidemic, disease severity is most accurately measured as percentage leaf area diseased and percentage of stem length affected. As disease increases, the simplest measure is percentage of leaves senesced and percentage of stems girdled (Wroth and Khan 1999). Disease scores tend to be high on the lower parts of the plant and low on the upper parts and therefore scores on the intermediate parts give a better comparison between different treatments or genotypes (Tivoli *et al.* 1996).

Since environmental factors contribute to variation in isolate reaction for *D. pinodes* (Wroth *et al.* 1998b), screening for host by pathogen interactions should be conducted in controlled conditions to reduce variability. The more aggressive isolates mask responses such as hypersensitivity while the least aggressive isolates produce the maximum variation in host response (Tivoli *et al. 2*007, Wroth *et al*. 1998b). Screening in controlled conditions for resistance should rely on isolates that produce moderate to severe lesions, and not aggressive isolates that could conceal partial resistance. Inoculum concentration needs to be intermediate for the same reason. Inoculum age, growing conditions and plant phenology also need to be taken into account when studying and identifying partial resistance (Onfroy *et al*. 1999, 2007, Tivoli *et al*. 2007).

1.8 Summary and aims of research

Ascochyta blight of field pea (synonym: blackspot) is widespread in Australian field pea crops and causes an estimated 15 % average loss of the national yield. The disease is complicated by having multiple causal organisms. Resistance to one pathogen, *A. pisi,* has

been incorporated into commercial cultivars of field pea around the world, but this has had little effect on disease control since no major gene resistance to *D. pinodes* or *P. medicaginis var. pinodella* has been found in the *P. sativum* germplasm, despite worldwide research. A fourth pathogen has also been identified as part of the complex in southern Australia but little is known about its biology or significance.

The control of ascochyta blight in field pea is reliant upon management practices that reduce the severity of the disease. Farmers in Australia have been advised to plant field pea after the majority of airborne ascospores of *D. pinodes* released from infested stubble have been depleted. However delayed sowing may compromise yield through shortening of the growing season. Recent research in Western Australia has modelled the release pattern for ascospores of *D. pinodes* over summer and autumn months ('G1 Blackspot Manager'). Recommendations have been made that crops can be sown when the model predicts that more than 60 % of ascospores have been released. The ascospore dispersal model has been validated in the main field pea-cropping region in Western Australia and two sites in South Australia. However, no research has been conducted to determine whether the sowing recommendations should be varied according to rainfall zones, or whether the absolute spore numbers vary per season and what impact this might have on the development of ascochyta blight epidemics. These issues should be resolved before 'G1 Blackspot Manager' is implemented in South Australia where field pea cropping occurs at a higher intensity than Western Australia.

The pathogens are dependent upon rainfall for both primary and secondary spore release and spread, leading to less severe infection in crops grown in lower rainfall regions. The dependency upon rainfall would suggest that the amount of primary inoculum can be higher in a lower rainfall crop since the disease will not spread as quickly in this situation. Epidemiological studies are required to investigate the link between primary inoculum and

resulting disease epidemics in different agro-ecological zones to maximise the benefits from 'G1 Blackspot Manager'.

Foliar fungicides are generally uneconomic against ascochyta blight except in high yielding crops, due to the low market value of field pea. Recent improvements in Australian cultivars have made fungicides economic in high to medium rainfall regions, provided the crops yield at least 2.5 t ha^{-1}. Higher yielding genotypes with earlier and longer flowering periods have recently been developed in the Australian field pea breeding program and optimal management strategies, as well as strategic foliar fungicides, need to be identified to maximise yields and economic returns in these plant lines.

Primary inoculum may also come from the soil-borne phase of all the causal pathogens, except for *A. pisi*. Farmers have been advised to maintain rotations of 5 - 6 years between field pea crops to minimise the soil-borne inoculum. The long-term soil-borne nature of *P. medicaginis* suggests that it may play a greater role where field pea is grown frequently. For the same reason it is possible that the unidentified *Phoma* may also be significant in this situation.

The aims of the research presented in this thesis were to: i) determine best disease management practice for the new field pea cultivars in a range of rainfall environments; (ii) describe the unidentified *Phoma* species and understand its role and significance in the ascochyta blight complex, and iii) determine the relationship between the amount of primary inoculum of *D. pinodes* and disease severity.

1.9 Linking statement

The research in this thesis is presented in seven chapters, including five research chapters, four of which have been published in peer reviewed journals and the fifth (Chapter 6) was accepted for publication on 23rd December 2012. The manuscripts are presented in order of date of publication.

Chapter 1 consists of an introduction to the thesis and a review of the literature of ascochyta blight of field pea. A summary of the literature and aims of research are presented at the end of this first chapter.

In Chapter 2 the new species of *Phoma* is identified as a part of the ascochyta blight complex on field pea in South Australia. Comparisons are made with the recognised causal pathogens of ascochyta blight, viz. *D. pinodes, P. medicaginis* var. *pinodella* and *A. pisi*. The findings reported in this paper were taken into account in all subsequent chapters, providing the rationale for positioning it as the first research chapter.

Chapter 3 presents research on the management of ascochyta blight in field pea, incorporating different sowing dates, fungicide strategies and germplasm with differing flowering dates. The influence of climate, represented by three growing regions in South Australia, on yield of field pea and on ascochyta blight was also reported in this chapter. Aspects of the disease management field trials in this manuscript are included in the following chapters, providing the rationale for positioning it as the second research chapter.

Research on the distribution and survival of the new species of *Phoma*, compared to *D. pinodes* and *P. medicaginis* var. *pinodella*, is presented in Chapter 4. Data for this study were collected from the disease management trials described in Chapter 3 and from other agronomic trials across the field pea growing districts of Australia.

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In Chapter 5 the cultural growth and *in planta* quantification of the new species of *Phoma* are compared to that of *D. pinodes* and *P. medicaginis* var. *pinodella*. Some of the data for this study were collected from the disease management trials described in Chapter 3.

D. pinodes is considered the principal pathogen in the ascochyta blight complex, due to the airborne ascospores that develop on senescent field pea plants, and hence was chosen for more in depth work regarding disease management. Chapter 6 presents research on the relationship between numbers of ascospores of *D. pinodes* released from infested pea stubble, secondary inoculum produced in canopies of different sowing dates and disease in the disease management trials described in Chapter 3. Crop management practices and their influences on ascochyta blight were also investigated in one district in South Australia.

A general discussion of the research in this thesis is presented in Chapter 7. Logical flow of the discussion determined that the findings on the new species of *Phoma* and comparisons with the other causal pathogens of ascochyta blight on field pea (Chapters 2, 4 and 5) are presented first, and the two chapters on disease management (Chapters 3 and 6) are subsequently discussed.

Chapter 2.

A new species of *Phoma* causes ascochyta blight symptoms on field peas (*Pisum sativum*) in South Australia.

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A new species of *Phoma* causes ascochyta blight symptoms on field peas (*Pisum sativum*) in South Australia

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Abstract: *Phoma koolunga* sp. nov. is described, having been isolated from ascochyta blight lesions on field pea (*Pisum sativum*) in South Australia. The species is described morphologically and sequences of the internal transcribed spacer region compared with those of the accepted pathogens causing ascochyta blight of field peas. *P. koolunga* was distinct from *Mycosphaerella pinodes* (anamorph: *Ascochyta pinodes*), *Phoma medicaginis* var. *pinodella* and *Ascochyta pisi*. Under controlled conditions the symptoms on pea seedlings caused by *P. koolunga* were indistinguishable from those caused by *M. pinodes*, other than a 24 h delay in disease development. Isolates of *P. koolunga* differed in the severity of disease caused on pea seedlings.

Key words: *Ascochyta pisi*, internal transcribed spacer (ITS), *Mycosphaerella pinodes*, *Phoma medicaginis* var. *pinodella*, systematics

INTRODUCTION

The recognized causal agents of ascochyta blight on field pea (*Pisum sativum*) are *Mycosphaerella pinodes* (anamorph *Ascochyta pinodes*), *Phoma medicaginis* var. *pinodella* (syn. *Phoma pinodella*) and *Ascochyta pisi* (Bretag and Ramsey 2001). Although these pathogens exist independently they often occur together within one pea field and even on single plants (Hare and Walker 1944). In South Australia *M. pinodes* and *P. medicaginis* var. *pinodella* are common in pea crops whereas *A. pisi* is rarely found (Ali and Dennis 1992). A fourth fungal species also has been isolated from ascochyta blight lesions on field peas in this region, and it originally was mis-identified as *Macrophomina phaseolina* (Ali and Dennis 1992). Field peas inoculated with this pathogen in glasshouse studies develop symptoms that are similar to those caused by the recognized ascochyta blight fungi (Ali and Dennis 1992).

In the current study DNA sequences of the internal transcribed spacer (ITS) region of this pathogen were compared with those of the recognized ascochyta blight causal pathogens and with *M. phaseolina* isolates from peanut (*Arachis hypogea*) and mung bean (*Vigna radiata*). The morphology of the pathogen is described. It was inoculated onto pea seedlings in controlled conditions, and disease expression was compared with symptoms caused by *M. pinodes* and *P. medicaginis* var. *pinodella*.

MATERIALS AND METHODS

Fungal isolation.—Field pea plants with typical ascochyta blight leaf and stem lesions were collected from commercial fields and trial sites across the cropping regions of South Australia (32–37°S, 134–140°E), 1995–2007. Diseased leaves or stem pieces were surface sterilized by dipping in 70% ethanol, followed by 30 s in 1% hypochlorite then rinsed in sterile water. They were placed onto potato dextrose agar (PDA) (Oxoid) amended with 0.01% streptomycin and plates were incubated 10–14 d under fluorescent lights (two Phillips TLD 36W/840 daylight tubes and one NEC black fluorescent light) for 12 h day/night at 22 C. The resulting isolates were identified as *M. pinodes*, *A. pinodes*, *P. medicaginis* var. *pinodella* and *Phoma koolunga* sp. nov., based on the morphological characteristics of the conidia and cultures. Single conidium-derived isolates were prepared and stored in sterile water at 4 C.

DNA extraction, sequencing and analysis.—DNA was extracted from 29 single conidium-derived cultures collected from field peas grown in South Australia, comprising 13 *P. koolunga* (TABLE I, FIG. 1), eight *M. pinodes*, one *A. pinodes*, five *P. medicaginis* var. *pinodella* and one *A. pisi* isolates. DNA also was extracted from two single conidium-derived

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TABLE I. Collection details and conidial measurement of *P. koolunga* isolates in these studies

Isolate identification number	Isolate used in pathogenicity study	Isolate used in ITS sequencing study	Isolate used for morphological description[1]	Collection site in South Australia (see FIG. 1)	Collection year	Average length of 100 conidia µm (s.d.) [2] on PDA	Average width of 100 conidia µm (s.d.) on PDA	% Oblong vs. % Ellipsoid
108/95		√		1. Sandergrove	1995	—	—	—
DAR 78534	√	√	√	2. Pinnaroo	1995	15.6(3.0)	7.4(2.6)	83:17
135/03		√		3. Blyth	2003	14.1(2.2)	8.2(2.2)	60:40
136/03	√	√		4. Yeelanna	2003	14.2(1.9)	5.6(1.3)	96:4
137/03		√		5. Wudinna	2003	—	—	—
138/03		√		6. Warrachie	2003	16.0(3.3)	8.1(2.3)	73:27
139/03	√	√		1. Sandergrove	2003	15.7(3.4)	8.0(2.4)	72:28
DAR 78533		√	√	7. Minnipa	2003	—	—	—
141/03		√		7. Minnipa	2003	—	—	—
142/03		√		7. Minnipa	2003	—	—	—
144/03	√	√		8. Balaklava	2003	13.9(2.2)	6.4(2.1)	91:9
147/03		√		9. Riverton	2003	—	—	—
DAR 78535	√	√	√	7. Minnipa	2004	14.5(2.6)	7.6(2.9)	73:27
81/06	√			10. Kingsford	2006	13.7(2.2)	8.6(3.1)	60:40
FT07010	√			11. Hart	2007	15.2(2.4)	5.6(1.5)	94:6
FT07013	√			7. Minnipa	2007	15.1(2.1)	6.5(1.6)	100:0
FT07019				12. Snowtown	2007	14.1(2.1)	7.7(2.4)	70:30
FT07022	√			13. Willamulka	2007	16.0(2.4)	5.4(1.1)	98:2
FT07025				8. Balaklava	2007	14.3(3.2)	7.4(2.2)	71:29
FT07026	√			9. Riverton	2007	15.0(2.1)	5.7(1.5)	95:5

[1] Two isolates (IMI 250052 = DAR67521 and IMI 250064 = DAR 67520) examined by Ali and Dennis (1992) also were included for the morphological description.
[2] Standard deviation.

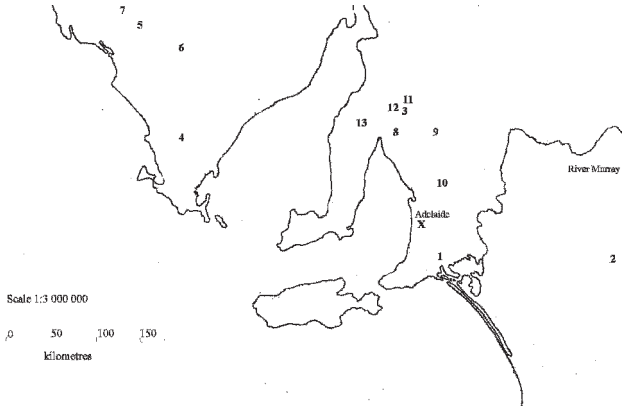

FIG. 1. Collection sites of 23 *Phoma koolunga* isolates representing geographic and temporal diversity.

isolates of *M. phaseolina*, collected from peanut (*A. hypogea*) and mung bean (*V. radiata*) grown in Queensland, Australia. All isolates were grown on amended PDA as described above 10–14 d. DNA was extracted from hyphae of each isolate with a modified Ultraclean™ Microbial DNA Isolation Kit (MoBio Laboratories Inc., California). The hyphae were carefully scraped from PDA plates with sterile surgical blades and transferred to individual microbead tubes to which 300 µL of microbead solution and 50 µL of solution MD1 were added. The tubes were placed in a FastPrep® FP120 cell disrupter (Bio 101, California) at 5.5 m/s for 30 s. The extraction was continued as per the manufacturer's protocol.

PCR volumes were 50 µL containing 0.4 µM each of primer TW81 (5′GTTTCCGTAGGTGAACCTGC 3′) and AB28 (5′ATATGCTTAAGTTCAGCGGGT 3′) (Curran et al 1994), 200 µM each of dGTP, dATP, dCTP, dTTP, 2.5 mM $MgCl_2$, 2 µL DNA, 1× supplied buffer and 1 unit Taq (QIAGEN Taq PCR Core Kit). These cycling conditions were used: denaturation step for 2 min at 94 C; then 35 cycles of 1 min at 94 C, 1 min at 55 C and 1.5 min at 72 C followed by a final post-extension at 72 C. PCR amplicons were visualized by electrophoresis in 1% Tris-borate/EDTA agarose gels containing 10 µg/mL ethidium bromide and purified for sequencing with the QIAGEN QIAquick® PCR Purification Kit according to the manufacturer's protocol. For direct sequencing up to 4 µL of purified product was used according to the Beckman Coulter Dye Terminator Sequencing with Quick Start Kit. Both strands of each fragment were sequenced on a Beckman Coulter CEQ 8800 capillary sequencer.

Sequences were imported into freely available sequence manipulation software, BioEdit (Hall 1999). Sequences were aligned with Clustal W (Thompson et al 1994) and were checked by eye. Phylogenetic trees were produced with neighbor joining (Saitou and Nei 1987) in the TREECON (van de Peer and de Wachter 1993) program. Reference sequences for *Ascochyta* and *Phoma* species were retrieved from GenBank and included in the analysis (FIG. 2). Evolutionary distances were corrected with the parameters of Jukes and Cantor (1969), Kimura (1980) and Tajima and Nei (1984). *M. phaseolina* was considered too distant, only

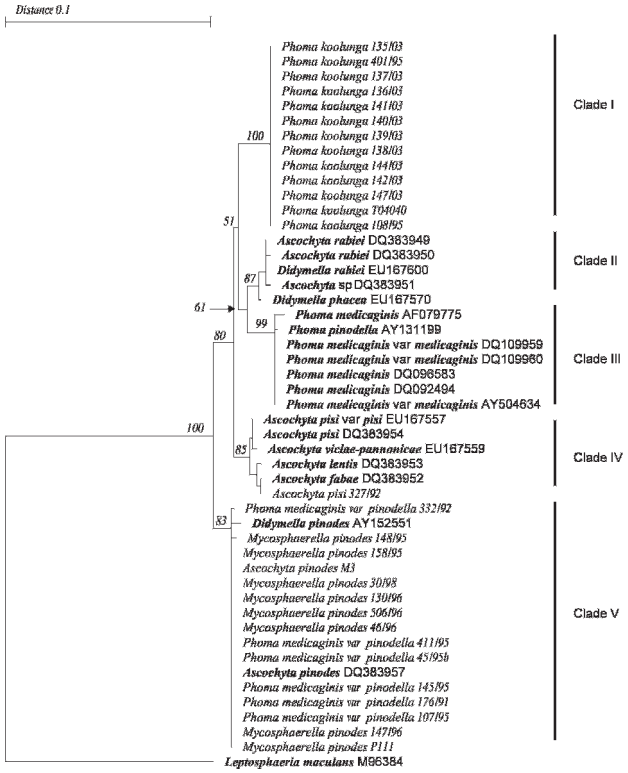

FIG. 2. Neighbor joining (Saitou and Nei 1987) tree of the ITS data constructed with Jukes and Cantor (1969) correction method. The tree was produced with TREECON (van de Peer and de Wachter 1993). Bootstrap values of 1000 replications are shown for the major nodes. Closest BLAST results were included in the analysis and are shown in boldface.

67% similar (data not shown) from *P. koolunga* to be considered as an outgroup, so *Leptosphaeria maculans*, 80% similarity, was chosen as a suitable sister taxon.

Morphological analysis.—Five representative single conidium-derived isolates of *P. koolunga* were used to describe the pathogen, including two isolates (IMI 250052 = DAR 67521 and IMI 250064 = DAR 67520) that were examined by Ali and Dennis (1992). The other three isolates, DAR 78535 (= T04040), DAR 78533 (= 140/03) and DAR 78534 (= 401/95), were included in the DNA sequence study above, and DAR 78535 also was used in subsequent pathogenicity studies (TABLE I). All isolates were cultured onto oat agar [Uncle Toby's Rolled Oats, 20 g boiled in 0.5 L water, strained before adding agar (15 g) and made up to 1 L with distilled water] and malt agar (BBL) and incubated in darkness for 7 days at 23 C and then under black light for a further 7–10 d under 12 h day/night (Boerema et al 2004). Colony diameter was measured at 7 d. Two diameters along perpendicular lines were measured for five plates of each medium. Characteristics of colonies on oat and malt agar were recorded. Pycnidial characters and conidial measurements were taken from colonies on oat agar. Pycnidial sections and conidia were mounted in lactic acid and 25 conidia from each isolate were measured under oil immersion at 1000× magnification. The colony and

conidial characteristics were compared with published descriptions of the ascochyta blight fungi and other similar *Phoma* species, as described by Boerema et al (2004).

Pathogenicity of three fungal species to pea seedlings.—Seeds of two field pea cultivars (Kaspa and Alma) and one breeding line (WAPEA2211) were sown into pots (11 × 11 × 14 cm) filled with pasteurized potting mix with nutrients. Kaspa and WAPEA2211 are erect semileafless (*afila*) types in which leaflets are replaced by tendrils whereas Alma is a conventional pea type. Each pea line was sown into 16 pots with four seeds per pot. Pots were placed in a controlled environment room (CER) as a randomized design with four replicates and were incubated at 15 C, 12 h day/night and watered as required until the seedlings had developed three complete nodes.

Cultures of *M. pinodes* (isolate FT07009), *P. medicaginis* var. *pinodella* (isolate FT07011) and *P. koolunga* (isolate DAR 78535) were grown 17 d on amended PDA as described in the fungal isolation procedure above. A conidial suspension of each pathogen was prepared by flooding the plates with sterile distilled water and gently rubbing the culture surface with a sterile glass rod to suspend the conidia. The concentration was determined with the aid of a haemocytometer and adjusted to 5×10^4 conidia per mL for each suspension, and surfactant Tween 20 (0.01%) was added. Each conidial suspension was sprayed onto four separate pots of each pea line until run-off, and control seedlings (four pots per pea line) were sprayed with sterile distilled water until run-off. After inoculation the seedlings were maintained in the CER at 20 C, 12 h day/night, in plastic tents (160 × 80 × 80 cm), each with an ultrasonic humidifier using reverse osmosis (RO) water to maintain leaf wetness. The seedlings were observed each day for symptom development and percent area diseased was assessed on the three lowest leaves (%LAD) and internodes (%IAD) on the seventh day. Data for %IAD for all four pea lines were combined for analysis. All data were square root-transformed and subjected to analysis of variance with GenStat version 10. When F values indicated significant differences, mean separation was based on least significant differences at 5% level of probability. Pathogens were re-isolated from representative lesions of each pathogen × pea line × replicate with the techniques described previously.

Pathogenicity of P. koolunga *on pea seedlings.*—Fourteen single conidium-derived isolates of *P. koolunga* in the collection were selected to represent geographical and temporal variation. These were grown on amended PDA 10–14 d as described above. The length and width of 100 conidia per isolate were measured with a light microscope at 200× magnification to compare with published descriptions of *M. pinodes*, *P. medicaginis* var. *pinodella* and *A. pisi*. Conidia were defined as oblong (conidium length equal to or more than 1.5× the width) or ellipsoid (conidium length less than 1.5× the width) and the ratio of oblong to ellipsoid conidia was calculated for each isolate. Ten of the 14 isolates, selected as representative (TABLE I, FIG. 1), were inoculated onto four pea lines with the methods described above. This experiment was restricted to 10 isolates due to space limitations in the CER. The lines comprised three field pea cultivars (Kaspa, Alma and Parafield, a conventional pea type) and one breeding line (WAPEA2211) sown into pots so that each pea line consisted of 44 pots with four seeds per pot. Disease data were analysed as above with untransformed data for %LAD and square root-transformed data for the %IAD.

RESULTS

Sequencing of internal transcribed spacer (ITS).—All *P. koolunga* isolates in this experiment were identical in ITS sequence and all grouped into one clade (clade I, FIG. 2). This clade appeared more homogeneous (bootstrap value = 100) than all other clades, and it contained only *P. koolunga*. The other clades contained more than one species, except possibly clade III. The neighbor joining tree constructed using the parameters of Jukes and Cantor (1969) showed each of the clades (I–V) individually supported by high bootstrap values (83% or more) while moderate bootstrap values supported the differentiation of clades I, II and III (51%). Trees produced by the methods of Kimura (1980) and Taijima and Nei (1984) gave similar results. GenBank accession numbers for the *P. koolunga* sequences are EU338415 to EU338444.

Taxonomy.—Comparison with published descriptions of the ascochyta blight fungi (TABLE II) distinguished *P. koolunga* from the other ascochyta blight pathogens on the basis of lighter mycelial color on malt agar, growth pattern on culture media and by the comparatively large, aseptate conidia. *M. pinodes* conidia are 8–16 × 3–4.5 μm, septate and constricted at the septum (Punithalingham and Holliday 1972). Colonies on culture media are light to dark gray, with pycnidia and pseudothecia distributed along the radii of mycelia growing out from the central point (FIG. 3A) (Onfroy et al 1999). Conidia of *A. pisi* are 10–16 × 3–4.5 μm, also with a constricted septum (Punithalingham and Gibson 1972). The conidia of *P. medicaginis* var. *pinodella* are aseptate 4.5–9 × 2–3 μm (Punithalingham and Gibson 1976), smaller than conidia of *P. koolunga*. Unlike colonies of *P. koolunga* (FIG. 3C), colonies of *P. medicaginis* var. *pinodella*, grown under a 12 h photoperiod of white light, form alternate zones of mycelium and pycnidia leading to concentric rings around the original mycelial plug (FIG. 3B) (Onfroy et al 1999).

The large, aseptate conidia produced by *P. koolunga* in culture are similar to those depicted in published descriptions of *M. phaseolina*. However the size of conidia of *M. phaseolina*, 14–30 × 5–10 μm (Holliday and Punithalingham 1970), is larger than that of the conidia of *P. koolunga* seen in this study.

TABLE II. Cultural and morphological characteristics of *Phoma koolunga* compared with published descriptions of ascochyta blight fungi of field pea

	Phoma koolunga	*Mycosphaerella pinodes*[bc]	*Phoma medicaginis* var. *pinodella*[cd]	*Ascochyta pisi*[ef]	*Macrophomina phaseolina*[g,h,i,j]
Colony color[a]	White-gray	Light to dark gray	Felty gray brown to black	Light brown	White to brown or gray, darker with age
Pycnidial distribution	Scattered	Radial and concentric	Concentric	Abundant	Few in culture
Conidia size in μm	12.5–17 × 5–7	8–16 × 3–4.5	4.5–8 × 2–3	10–16 × 3–4.5	14–30 × 5–10
Conidial septation	Mostly aseptate	1-, occasionally 2-, septate, constricted at septum	Mostly aseptate	1-septate, constricted at septum	Aseptate
Conidial exudate	Pale creamy white	Light buff to flesh colored	Light buff	Carrot red on oat agar	Dull white to cream

[a] Colony color on malt agar as seen from above.
[b] Punithalingham and Holliday (1972).
[c] Onfroy et al (1999).
[d] Punithalingham and Gibson (1976).
[e] Punithalingham and Gibson (1972).
[f] Bretag and Ramsey (2001).
[g] Holliday and Punithalingham (1970).
[h] McGee (1991).
[i] Sutton (1980).
[j] Ahmed and Reddy (1993).

Pycnidia of *M. phaseolina* rarely develop in culture (Holliday and Punithalingham 1970, Sutton 1980) while those of *P. koolunga* develop readily. The pycnidia of *M. phaseolina* always are described as dark brown to black (Ahmed and Reddy 1993, Holliday and Punithalingham 1970, McGee 1991, Sutton 1980) but pycnidia of *P. koolunga* are initially pale yellow to brown.

P. koolunga belongs in *Phoma* section *Macrospora* as defined by Boerema et al (2004), due to its large conidia, which occasionally become 1-septate. Only two species in this section have been described from leguminous plants, *P. boeremae* Gruyter from stems and seeds of *Medicago* spp. and *P. rabiei* (Pass.) Kovatsch. ex Gruyter the cause of blight of chickpea. The conidia of both those species are 3.5–5.5 μm wide, which generally is narrower than those of *P. koolunga* (5–7 μm).

Phoma koolunga Davidson, Hartley, Priest, Krysinska-Kaczmarek, Herdina, McKay & Scott sp. nov.

Pycnidia in vitro 110–210 μm diam, globosa, solitaria vel confluenta, glabra vel pilosi brevissima, 1–3 ostiolis papillatis, mellea- brunnea vel olivacea- nigra. Cellulae conidiogenae globosae 10–13 × 8–12 μm. Conidia hyalina, ellipsoidea vel oblongata, 12.5–19.5 × 5–7 μm, aseptata, pauca conidia uniseptate.

Holotypus. Cultura exsiccate in agaro isolatus e *Pisum sativum*, Minnipa, South Australia (32°51′S 135°09′E altitude 151 m), Meridionalis Nova Hollandia, Nova Hollandia, J. Davidson (T04040), 26 Oct 2004 (DAR 78535).

Etymology. From the native Australian word for the district north of Adelaide where field peas are widely grown.

Colony on oat agar 13–20 mm diam after 7 d, regular with sparse, creamy-white through gray to yellow brown aerial mycelium, colony yellow-brown to pale olivaceous, reverse yellow-brown. Malt agar: colony 7–12 mm diam after 7 d, irregular with compact finely cottony white to pale gray aerial mycelium, colony mostly gray-white, occasionally dark olivaceous with little or no aerial mycelium, reverse yellow-brown to olivaceous. *Pycnidia* scattered over the agar or immersed, (110–)150–210 μm diam, globose often with an elongated neck, solitary, occasionally confluent, glabrous or more often covered by hyphal outgrowths or short mycelial hairs, with 1(–3) papillate ostioles, pale yellow-brown at first becoming dark olivaceous black, with four or five cell wall layers composed of pseudoparenchymatous cells, the outer layer being thickened and pigmented. Conidial ooze pale creamy-white. *Conidiogenous* cells globose, 10–13 × 8–12 μm. *Conidia* hyaline, ellipsoidal to oblong, 12.5–17(–19.5) × 5–7 μm, mostly aseptate with a few 1-septate conidia

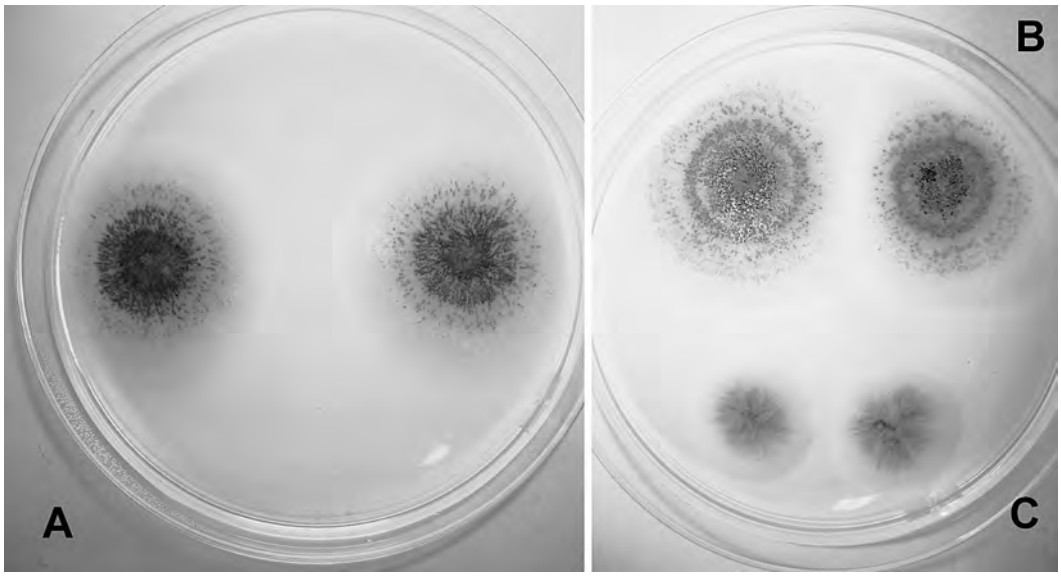

FIG. 3. Cultures of (A) *Mycosphaerella pinodes*, (B) *Phoma medicaginis* var. *pinodella* and (C) *Phoma koolunga* on PDA, incubated 10–14 d under fluorescent light (two Phillips TLD 36W/840 daylight tubes and one NEC black fluorescent light) for 12 h day/night at 22 C.

being present (FIG. 4). *Chlamydospores* absent but pseudosclerotia may be present, often formed on radiating lines of pale yellow-brown to dark brown mycelium. *Crystals* absent.

Cultures examined. All from *Pisum sativum*, South Australia; Freeling 1976, *M. Ali* 28 (IMI 250052, DAR 67521); Maitland, 1978, *M. Ali* (IMI 250064, DAR 67520); Minnipa, 2003, *J. Davidson* (DAR 78533); Pinnaroo 1995, *J. Davidson* (DAR 78534); Minnipa, 26 Oct 2004, *J. Davidson* (DAR 78535) *type*.

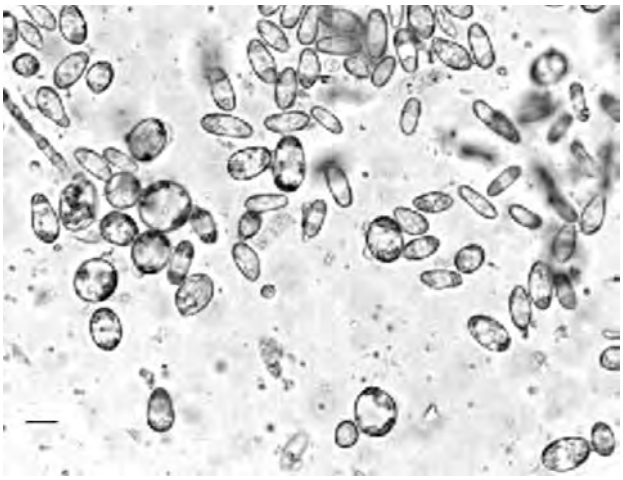

FIG. 4. Variation in shape of conidia of *Phoma koolunga*, produced on PDA after incubation 10–14 d under fluorescent light (two Phillips TLD 36W/840 daylight tubes and one NEC black fluorescent light) for 12 h day/night at 22 C, photographed at 200× magnification with a light microscope. Bar = 10 μm.

Pathogenicity of three fungal species on pea seedlings.— Twenty-four hours after inoculation the seedlings inoculated with *M. pinodes* had developed chlorotic spots 1 mm diam. By 48 h post-inoculation these had grown to 5 mm diam and were becoming necrotic. Seedlings inoculated with *P. koolunga* developed chlorotic and necrotic spots 1–3 mm diam by 48 h post-inoculation. Necrotic lesions continued to expand at a similar rate, so that lesions caused by *M. pinodes* remained slightly larger than those caused by *P. koolunga* for the duration of the experiment. Symptoms caused by the two pathogens were identical (FIG. 5). Seedlings inoculated with *P. medicaginis* var. *pinodella* developed necrotic lesions more slowly, and no symptoms were observed until 72 h post-inoculation. These lesions remained at 1 mm diam or less for the duration of the experiment.

The interaction between pathogen and pea lines was significant for %LAD. There was significantly ($P = 0.02$) more %LAD on Kaspa than on the other two pea lines when inoculated with *M. pinodes*. Likewise, disease on leaves of Kaspa was significantly ($P = 0.02$) more severe than that on WAPEA2211 when inoculated with *P. koolunga* (TABLE III). The interaction between pathogen and pea line was not significant for %IAD, hence the data for the four pea lines were combined. The %IAD was significantly ($P < 0.001$) greater for plants inoculated with *M. pinodes* than *P. koolunga* (TABLE III). The pathogens that were inoculated onto the seedlings were re-isolated from each of the representative lesions and their identity confirmed, satisfying Koch's postulates.

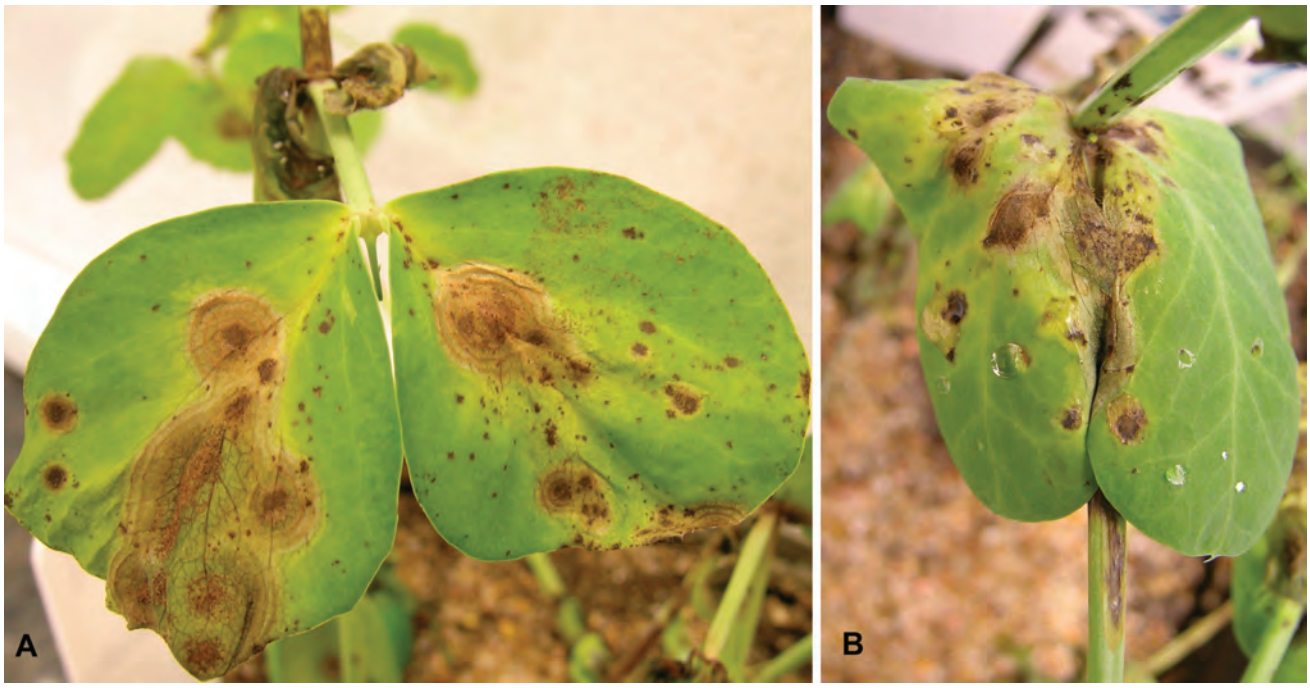

FIG. 5. Symptoms on field pea seedlings inoculated with (A) *Mycosphaerella pinodes*, (B) *Phoma koolunga*, 7 d post-inoculation.

Pathogenicity of P. koolunga *on pea seedlings.*—The average size of the conidia of the 14 *P. koolunga* isolates cultured on PDA was 13.7–16.0 × 5.5–8.6 µm. The majority of the conidia were oblong however the isolates produced varying proportions of conidia that were ellipsoid (TABLE I). All seedlings inoculated with the 10 representative *P. koolunga* isolates developed chlorotic and necrotic spots 1–3 mm diam by 48 h post-inoculation, whereas controls remained healthy. The interaction between isolates and pea lines was significant ($P = 0.05$) for the %LAD and the %IAD. At 7 d post-inoculation the %LAD for each combination of isolate × pea line was 16.8–94.8% (FIG. 6) and the %IAD was 1.8–3.2 (square-root) (FIG. 7) (untransformed data: 3.5–11%). Isolate DAR 78535 caused the least severe disease on leaves on all four pea lines and FT07013 caused severe disease on leaves on all four pea lines. Both DAR 78535 and FT07013 were isolated from field pea plants at Minnipa, although in different years. The oldest isolate in this experiment, DAR 78534, caused moderate to severe disease.

DISCUSSION

Phoma koolunga was isolated from typical ascochyta blight lesions on field pea collected from the pea growing zone of South Australia, and 29 cultures were placed in storage 1995–2007. DNA sequencing

TABLE III. Disease severity on leaves and internodes (square root-transformed) on pea seedlings incubated in controlled environment conditions assessed 7 d after inoculation

	Control	*Mycosphaerella pinodes*	*Phoma koolunga*	*Phoma medicaginis* var. *pinodella*
		Average percent leaf area diseased		
Pea line				
Alma	0.0(0.0)[a]	5.1(28.0)	4.3(20.4)	1.0(1.8)
Kaspa	0.0(0.0)	6.0(36.7)	4.8(24.3)	0.5(0.4)
WAPEA2211	0.0(0.0)	4.9(24.5)	4.0(16.6)	0.6(0.9)
LSD= 0.6				
Average percent internode diseased (data combined for all four pea lines)				
	0.0(0.0)	2.3(6.1)	2.1(4.8)	0.4(0.3)
LSD= 0.2				

[a] Untransformed data are in parentheses.

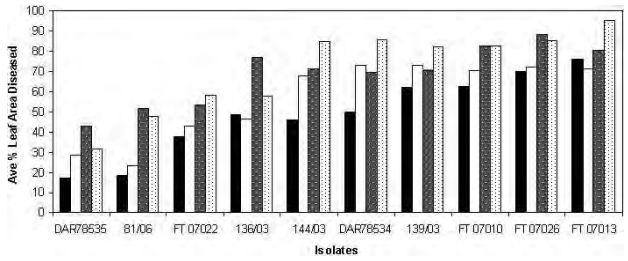

FIG. 6. Average percent leaf area diseased on four field pea lines (*Pisum sativum*), breeding line WAPEA2211 (■), cv. Kaspa (□), cv. Alma (◘), cv. Parafield (⊡), inoculated at the three-node growth stage with 10 single conidium-derived isolates of *Phoma koolunga*, 7 d post-inoculation. Least significant difference ($P = 0.05$) = 9.0.

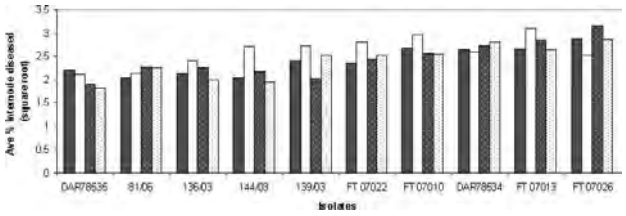

FIG. 7. Average percent internode diseased (square root) on four field pea lines (*Pisum sativum*), breeding line WAPEA2211 (■), cv. Kaspa (□), cv. Alma (◘), cv. Parafield (⊡), inoculated at the three-node growth stage with 10 single conidium-derived isolates of *Phoma koolunga*, 7 d post-inoculation. Least significant difference ($P = 0.05$) = 0.4.

of the ITS regions and morphological examination showed *P. koolunga* to be a unique species, distinct from the accepted causal pathogens of ascochyta blight of field pea (viz. *M. pinodes*, *P. medicaginis* var. *pinodella* and *A. pisi*). Results also showed the original identification of the fungus as *M. phaseolina* to be incorrect.

The taxonomy of *Ascochyta* spp. on legume crops is based on morphological characters such as shape and size of conidia, conidial septation, host from which isolated and molecular markers (Peever 2007). The comparatively large size of the conidia of *P. koolunga* and their aseptate nature and the production and color of pycnidia in culture distinguish the fungus from *M. pinodes*, *P. medicaginis* var. *pinodella* and *A. pisi*, and also from *M. phaseolina*. The variation in proportion of oblong and ellipsoid conidia in individual cultures is another distinguishing feature of *P. koolunga*.

The ITS sequences of *M. pinodes*, *P. medicaginis* var. *pinodella* and *A. pisi* are highly conserved within each species, even for isolates sampled from diverse locations worldwide (Peever et al 2007). Similarly *M. phaseolina* does not exhibit polymorphism in the ITS region (Su et al 2001). This consistency in the ITS region has been established for *P. koolunga* isolates from a wide geographical distribution across South Australia. Hence differences in ITS sequences strongly support a separate taxonomic grouping of *P. koolunga* and, in combination with the morphological data, provide evidence for a new species. The ITS sequences of all the *P. koolunga* isolates in this study were identical, supporting the choice of this region to differentiate among the fungal species.

Peever et al (2007) have differentiated *M. pinodes* and *P. medicaginis* var. *pinodella* through phylogenetic analysis of G3PD sequences. Studies of this and other protein-encoding genes, such as translation elongation factor 1-alpha (EF) and chitin synthase 1 (CH), may assist in determining the phylogenetic relationship of *P. koolunga*, *M. pinodes* and *P. medicaginis* var. *pinodella*.

The symptoms caused by *P. koolunga* were indistinguishable from those caused by *M. pinodes*, other than a 24 h delay in development. At 7 d post-inoculation the severity of disease was similar to that caused by *M. pinodes*. The limited lesion development on the leaves and internodes by *P. medicaginis* var. *pinodella* supported the observation by Onfroy et al (1999) that this pathogen is less aggressive than *M. pinodes*. However *P. medicaginis* var. *pinodella* was more aggressive than *M. pinodes* when inoculated onto pea epicotyls (Knappe and Hoppe 1995).

All 10 isolates of *P. koolunga* inoculated onto pea seedlings were pathogenic and caused extensive necrotic leaf and internode lesions. The isolates demonstrated a range in aggressiveness that was not linked to the site or year of collection.

The line WAPEA2211 had been selected for ascochyta blight resistance (T Khan pers comm). When this line was inoculated with *P. koolunga* or *M. pinodes*, disease on leaves was less severe than on the other lines tested. However in the isolate variability experiment, disease severity on leaves in general was similar on WAPEA2211 and Kaspa, both of which were significantly less than on Alma or Parafield. Further studies are needed to confirm that WAPEA2211, and perhaps Kaspa, are moderately resistant to *P. koolunga* and also to ascertain whether the putative resistance is heritable and whether the same resistance mechanism acts against *M. pinodes*.

Ali et al (1982) found *P. koolunga* (then misidentified as *M. phaseolina*) to be seed borne and present on 72% of the South Australian commercial seed lots tested. The severity of lesions caused by *P. koolunga* in controlled conditions indicated that this fungus might be an important component of ascochyta

blight in South Australian field peas. Studies of the distribution of *P. koolunga* across South Australia and pathogenicity on peas grown in field conditions are in progress.

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Chapter 3.

Combining management and breeding advances to improve field pea (*Pisum sativum* L.) grain yields under changing climatic conditions in south-eastern Australia.

Published article – *Euphytica* **180**, 69 – 88.

McMurray. L.S., Davidson, J.A., Lines, M.D., Leonforte, A. & Salam, M.U. (2011) Combining management and breeding advances to improve field pea (*Pisum sativum* L.) grain yields under changing climatic conditions in south-eastern Australia.
Euphytica, v. 180(1), pp. 69-88

Chapter 4.

Distribution and survival of ascochyta blight pathogens in field-pea-cropping soils of Australia.

Published article – *Plant Disease* **95**, 1217 – 1223.

Davidson, J.A., Krysinska-Kaczmarek, M., Wilmshurst, C.J., McKay, A., Herdina, & Scott, E.S. (2011) Distribution and survival of ascochyta blight pathogens in fieldpea - cropping soils of Australia. *Plant Disease, v. 95(10), pp. 1217-1223*

Chapter 5.

Comparison of cultural growth and in planta quantification of *Didymella pinodes*, *Phoma koolunga* and *Phoma medicaginis* var. *pinodella*, causal agents of ascochyta blight of field pea (*Pisum sativum*).

Published article – *Mycologia* **104**, 93 – 101.

Mycologia, 104(1), 2012, pp. 000–000. DOI: 10.3852/11-118

Comparison of cultural growth and in planta quantification of *Didymella pinodes*, *Phoma koolunga* and *Phoma medicaginis* var. *pinodella*, causal agents of ascochyta blight on field pea (*Pisum sativum*)

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Abstract: The causal agents of ascochyta blight on field pea in South Australia, *Didymella pinodes*, *Phoma medicaginis* var. *pinodella* and *Phoma koolunga*, are isolated from a single plant within a crop, suggesting competition for space and nutrients. Interactions among these pathogens were investigated. Diameters of colonies of *D. pinodes* and of *P. medicaginis* var. *pinodella* were significantly reduced on PDA amended with filtrate from broth cultures of *P. koolunga* as were diameters of colonies of *D. pinodes* on PDA amended with filtrate from *P. medicaginis* var. *pinodella* or *D. pinodes*. This effect was negated when cultures were transferred to unamended PDA, indicating filtrates were fungistatic instead of fungicidal. The diameter of *P. koolunga* colonies was not influenced by filtrate from any of the three species. When pathogens were co-inoculated in pairs onto leaves on field pea plants, the quantity of DNA of *D. pinodes* and of *P. medicaginis* var. *pinodella* was significantly reduced if co-inoculated with *P. koolunga*. The quantity of DNA of *P. koolunga* was not influenced by co-inoculation. When co-inoculated onto excised leaf disks on sterile water the mean lesion diameter due to *D. pinodes* and to *P. medicaginis* var. *pinodella* was significantly reduced if co-inoculated with *P. koolunga* isolate DAR78535. Lesions caused by *D. pinodes* were significantly reduced when inoculum was self-paired. Conversely the diameter of lesions caused by *P. koolunga* DAR78535 increased when self-paired or when co-inoculated with *P. medicaginis* var. *pinodella*. Unlike leaf disks on sterile water, co-inoculation had no influence on lesion size or quantity of pathogen DNA in leaf disks on water agar. Antagonism, including self-antagonism, was detected among these species, leading to reduction in lesion size and quantity of pathogen DNA. The slower growing species, *P. koolunga*, was not self-antagonistic, and in a few instances the effect of co-inoculation was additive or synergistic.

Key words: fungicidal, fungistasis, *Mycosphaerella pinodes*

INTRODUCTION

The fungal pathogen *Phoma koolunga* has been identified as an important component of the ascochyta blight complex on field pea (*Pisum sativum*) in South Australia where it is widespread in the field pea cultivation regions (Davidson et al. 2009a, 2011). *Didymella pinodes* (synonym: *Mycosphaerella pinodes*) (Peever et al. 2007) has a major role in the disease complex in this region (Bretag et al. 2006, Carter 1963, Carter and Moller 1961), and *Phoma medicaginis* var. *pinodella* is also commonly isolated whereas *Didymella pisi* is rarely detected in ascochyta blight-affected field pea plants in South Australia (Davidson et al. 2009a). The former three pathogens are regularly isolated from the same plants from the same or adjacent lesions on leaves, pods and stems (Bretag and Ramsey 2001; Davidson et al. 2009a, 2011). In South Australia *P. koolunga* and *D. pinodes* were isolated in equal proportions from naturally infected plants of cultivar (cv.) Kaspa, the most widely grown cultivar in Australia (McMurray et al. 2011), whereas *P. medicaginis* var. *pinodella* was detected at a much lower frequency (Davidson et al. 2011). In controlled conditions the pathogenicity of *P. koolunga* on cv. Kaspa was similar to that of *D. pinodes* while lesions caused by *P. medicaginis* var. *pinodella* were less than 1 mm diam (Davidson et al. 2009).

The three pathogens, *D. pinodes*, *P. medicaginis* var. *pinodella* and *P. koolunga*, can survive in soil for a number of years (Davidson et al. 2011, Wallen et al. 1967, Wallen and Jeun 1968) and often are colocated in the soil (Davidson et al. 2009b; 2011). The quantity of soilborne inoculum has been correlated with

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disease severity on field pea grown in controlled conditions (Davidson et al. 2009b). These pathogens also may survive as mycelia on organic matter (Bretag and Ramsey 2001).

The colocation of these pathogens on host plants and in soil suggests that there is competition among them for access to space and nutrients on the plant. Le May et al. (2009) reported both antagonistic and synergistic interactions between *D. pinodes* and *P. medicaginis* var. *pinodella* when co-inoculated onto individual pea plants, depending on location and timing of application of conidia. Competition between the two pathogens on the same leaf caused a reduction in lesion size, although synergistic or additive responses were noted when 3 or 6 d occurred between inoculations on different leaves of the same plant (Le May et al. 2009). *P. koolunga*, which has been reported only in southern Australia to date, was not included in that study. Davidson et al. (2009a) found that symptom development on field pea in controlled conditions was slower for *P. koolunga* than for *D. pinodes*, suggesting that the latter pathogen might have a competitive advantage if the two infect the same leaf. While it is common for plants to be infected by more than one species simultaneously, these three species appear to occupy the same niche on the host plant. It is possible that subtle differences in the timing or location of events or in resource use might allow coexistence (Fitt et al. 2006) or that they might compete for the same space and resources.

Ascochyta blight complex can cause significant yield loss in field pea crops in South Australia (McDonald and Peck 2009, McMurray et al. 2011), and fungicides are often uneconomic except in higher yielding crops (McMurray et al. 2011). Pulse Breeding Australia is attempting to improve resistance to this disease complex using minor genes because no major gene resistance has been found for *D. pinodes* or *P. medicaginis* var. *pinodella* (Bretag et al. 2006). Understanding the role of *P. koolunga* and the relative competitiveness and aggressiveness of the pathogens in this complex will assist in setting priorities in resistance breeding.

Preliminary experiments had identified zones of inhibition between colonies of *P. koolunga* and *D. pinodes* or *P. medicaginis* var. *pinodella* when co-inoculated onto potato dextrose agar (PDA), suggesting antifungal metabolites produced by the pathogens had diffused through the nutrient medium. Such metabolites may be fungicidal or fungistatic. A number of fungal species are known to produce a range of metabolites with detrimental effects on fungal pathogens. For example metabolites from *Trichoderma virens* were fungicidal against the pathogen *Phytophthora erythroseptica*, completely inhibiting growth even after the pathogen was transferred to metabolite-free medium. Growth of *P. erythroseptica* was reduced in the presence of metabolites of *T. harzianum* but resumed upon transfer to fresh medium, demonstrating a fungistatic effect (Etebarian et al. 2000). The aims of the current study were to investigate interactions among the pathogens *P. koolunga*, *D. pinodes* and *P. medicaginis* var. *pinodella* in nutrient medium and in planta and identify antagonistic or synergistic relationships.

TABLE I. Single conidium-derived isolates of *Phoma koolunga*, *Didymella pinodes* and *Phoma medicaginis* var. *pinodella* co-inoculated on agar medium and on plant material

Pathogen species	Isolate identification	Year of collection	Collection site
P. koolunga	DAR78535	2004	Minnipa[a]
P. koolunga	FT07006	2007	Turretfield[b]
D. pinodes	FT07005	2007	Turretfield
P. medicaginis var. *pinodella*	FT07007	2007	Turretfield

[a] Low rainfall (325 mm pa) region on Eyre Peninsula in South Australia.

[b] Medium-high rainfall (425 mm pa) region in mid-north agriculture region of South Australia.

MATERIALS AND METHODS

Comparison of colony growth on PDA amended with broth filtrate.—To determine whether the inhibition of mycelial growth observed in the preliminary experiment was fungistatic or fungicidal four isolates, *P. koolunga* DAR78535, *P. koolunga* FT07006, *D. pinodes* FT07005 and *P. medicaginis* var. *pinodella* FT07007 (TABLE I), were grown on medium amended with filtrates of broth cultures (Chambers 1992, Dennis and Webster 1971) as outlined below.

The single conidium-derived isolates of *P. koolunga*, *D. pinodes* and *P. medicaginis* var. *pinodella*, were obtained from typical ascochyta blight leaf and stem lesions from commercial pea fields and stored as mycelial plugs in sterile water at 4 C (Davidson et al. 2009a). Mycelial plugs were transferred from storage vials to PDA in 9 cm diam Petri dishes and incubated 10–14 d under fluorescent light and near ultraviolet light under 12 h/12 h dark/light cycles at 22 C. Conidial suspensions of individual isolates were prepared by flooding the plates with sterile reverse osmosis (RO) water and gently rubbing the culture surface with a sterile glass rod to suspend the conidia. The concentration was determined with the aid of a haemocytometer, adjusted to 1×10^5 conidia mL^{-1} for each suspension, and surfactant Tween 20 (0.01%) was added.

Sterilized yeast extract broth (10 g Bacto and 10 g sucrose in 1 L RO water) was poured into 20 Schott bottles (100 mL per bottle) and cooled to room temperature, after which 1 mL conidial suspension was added to each bottle. Each of the four isolates was added to four separate bottles, and an

extra four bottles without conidial suspension served as controls. The bottles were incubated at room temperature 7 d on a platform shaker at 100 rpm. The resulting mycelial cultures were passed through filter paper (Filtech, grade 1803, 90 mm diam) in a ceramic Buchner funnel (90 mm diam) and then through a glass fiber paper filter (Whatman, GF/A, 70 mm diam) in a Duran glass filter funnel (70 mm diam) using vacuum suction for each process. The funnels and vacuum bottles were cleaned between each bottle of broth culture by washing in sterile RO water, soaking in 0.95% hypochlorite (Milton® bleach) 3 min and rinsing in sterile RO water. The resulting filtrate was sterilized by passing through a 0.2 µm syringe filter (Whatman Puradisc 25 PES, 25 mm diam) on a 30 mL Terumo syringe. Due to time constraints the filtrates were refrigerated at 4 C overnight. The filtrate from each of the four isolates or sterile broth (control), 25 mL, was added to 75 mL molten PDA (3.9 g PDA in 75 mL sterile RO) and poured into 9 cm diam Petri dishes (16 dishes per preparation). A mycelial plug (3.0 mm diam) of a test isolate was placed in the center of each amended PDA plate. The four isolates and four lots of amended PDA, as well the unamended control, were tested in all possible combinations, replicated four times. Petri dishes were sealed with parafilm, incubated 7 d as described above, and the diameter of the resulting colonies was measured at 3 d and 7 d. To test whether any effects were fungicidal or fungistatic the inoculum plugs from each were removed from the amended PDA after 7 d, transferred to unamended PDA and incubated as before. Colony diameter was measured after 3 and 7 d incubation. No growth indicated a fungicidal effect, but resumption of normal growth indicated a fungistatic effect. The experiment was repeated as above but without overnight refrigeration of the sterile filtrate to determine whether refrigeration might have modified the activity of the filtrate.

Comparison of lesion size and quantity of pathogen DNA on leaves of intact plants.—Seeds of field pea cv. Kaspa were sown into 120 pots (8.5 × 7 × 6 cm) filled with pasteurized University of California potting mix with nutrients, two seeds per pot, placed in a controlled environment room (CER) at 16 C, 12 h/12 h light/dark cycle and watered as required. When seedlings developed three complete nodes they were thinned to one plant per pot. Spore suspensions of *P. koolunga* isolates DAR78535 and FT07006, *D. pinodes* isolate FT07005 and *P. medicaginis* var. *pinodella* isolate FT07007 (TABLE I) were prepared as described above. These isolates were paired in all possible combinations by inoculation of the second leaf with droplets of spore suspension as described by Le May et al. (2009), eight replicate plants per combination. However this was unsuccessful due to the hydrophobic surface of leaves of cv. Kaspa, so the following method was adopted. A 3 mm diam mycelial plug of the first isolate was placed on the second youngest leaf and a similar plug of the second isolate was placed on the same leaf approximately 1 cm distant. Controls consisted of 3 mm diam plugs of sterile PDA paired with mycelial plugs of the test isolates. After inoculation the seedlings were maintained in the CER at 20 C, 12 h day/night in plastic tents (160 × 80 × 80 cm), each with an ultrasonic humidifier using RO water to maintain leaf wetness. The seedlings were observed each day for symptoms, and lesion diameter including chlorosis was assessed 3 d post inoculation (p.i.). Where lesions had partially merged the diameter of each paired lesion could be measured whereas the few cases where lesions had totally merged were treated as missing data. Inoculated leaves were collected from four replicate plants for each pair of isolates and control pair, and agar plugs were removed. The leaves were placed in individual screw cap tubes (1.5 mL) (Astral Scientific, Australia) with pin-prick holes in aluminum foil closures for ventilation and stored at −20 C before lyophilizing approximately 3 wk later. The remaining plants were incubated until 7 d p.i., at which point they were assessed for lesion diameter and stored as described above.

Leaves were lyophilized with a Cuddon FD80, and DNA was extracted with a SARDI DNA extraction kit (Herdina et al. 2004, Riley et al. 2009). The quantity of pathogen DNA per leaf was estimated with real-time quantitative PCR (qPCR). The primers and probes for these assays were designed based on the highly conserved internal transcribed spacer (ITS) region of the ribosomal genome (Davidson et al. 2009a, Ophel-Keller et al. 2008). *D. pinodes* and *P. medicaginis* var. *pinodella* are identical in the ITS region (Peever et al. 2007), and the same DNA test was used to quantify these two pathogens, designated *DpPmp*. A separate DNA test was used to quantify DNA of *P. koolunga*. Both assays were designed with sequence information from local isolates and from GenBank, and specificity was evaluated with a range of target and closely related nontarget isolates (Davidson et al. 2009a). The preparation of DNA standards and PCR conditions were as described by Riley et al. (2009).

Comparison of lesion size and quantity of pathogen DNA on excised leaf disks.—Seedlings (120) of field pea (cv. Kaspa) were grown to three nodes as described above. Leaves from the second node were collected and 1.4 cm diam disks were cut from each leaf with a sterile brass cork borer, after marking the stem side of the disk with a pen. Immediately after cutting the disks were placed, adaxial side up, into Petri dishes (3.5 cm diam) containing 4 mL sterile RO water, one disk per dish. Conidial suspensions of the four isolates were prepared as described above. Isolates were paired in all possible combinations on the disks, eight replicate leaves per treatment, as follows. A 10 µL droplet conidial suspension of the first isolate was placed on the disk, and a 10 µL droplet of the second isolate was placed approximately 5 mm distant. Controls consisted of a 10 µL droplet sterile RO water paired with a 10 µL droplet conidial suspension of the test isolates. The inoculum remained in place due to horizontal position of the disks, in contrast to that on intact plants. The disks were incubated under fluorescent light and near ultraviolet light for 12 h dark/light cycles at 22 C with lids closed but not sealed. At 3 d p.i. the diameter of the lesions on four replicate leaves per isolate pair was measured and the disks were placed in individual tubes as above. The remaining disks were incubated until 7 d p.i., then lesion diameter was assessed.

Leaves were stored and the quantity of pathogen DNA in each disk was estimated as described above. This experiment was repeated with the disks supported on 0.5% water agar instead of floating on sterile RO water.

Statistical analysis.—Analysis was conducted with Genstat 12.1. The two broth filtrate experiments, with and without overnight refrigeration, were analyzed together with REML linear mixed models and analyzed separately with analysis of variance (ANOVA) for randomized blocks. Data from co-inoculation experiments on plants and detached disks were compared with ANOVA for randomized blocks. Data from DNA assays were logarithm-transformed for all analyses. When F values indicated significant differences, mean separation was based on least significant differences at 5% of probability.

RESULTS

Comparison of colony growth on PDA amended with broth filtrates.—The three-way interaction between experiments × isolate × filtrate was significant ($P < 0.002$) at 3 d p.i. on amended agar. This interaction was due to *P. medicaginis* var. *pinodella*, which grew slowly in the first experiment (where filtrate was refrigerated) when paired with itself, with *D. pinodes* or the control, whereas colony diameter of this pathogen when paired with *P. koolunga* was similar in both experiments. The diameters of colonies in all other pairwise combinations were similar in both experiments at this time of assessment. The two-way interactions (experiments × isolates, experiments × filtrates, isolates × filtrates) all were significantly ($P < 0.05$) different at 7 d p.i. on amended PDA while at 3 d p.i on normal PDA the two-way interaction between experiments × isolates also was significant ($P < 0.001$). No significant differences between experiments were identified at 7 d p.i. on normal PDA. These interactions suggest that storing the filtrate overnight at 4 C might have affected results. Hence the results of the second experiment, in which the filtrate was not refrigerated, were analyzed separately and are presented (FIG. 1). All cultures of *P. medicaginis* var. *pinodella* were significantly ($P < 0.001$) larger than those of *D. pinodes*, which in turn were significantly ($P < 0.001$) larger than those of *P. koolunga*, irrespective of medium. After 3 d incubation the mean diameters of *D. pinodes* and *P. medicaginis* var. *pinodella* were significantly ($P < 0.002$) reduced 22–40% on medium amended with filtrate of one or both of the *P. koolunga* isolates (FIG. 1A, B). At the same time the diameter of *D. pinodes* also was reduced by 24% (FIG. 1A) on medium amended with filtrate of *P. medicaginis* var. *pinodella*. After 7 d incubation the mean diameter of *D. pinodes* was reduced 10–19% (FIG. 1A) when grown on medium amended with filtrate from any of the

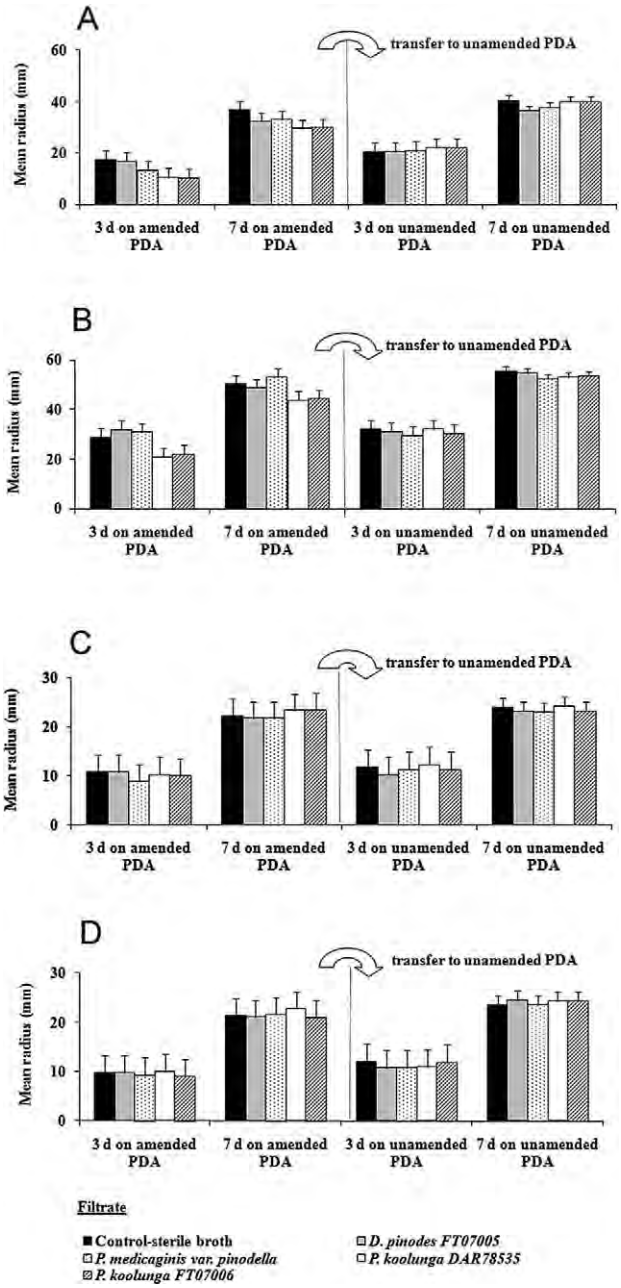

FIG. 1. Mean diameter of colonies of (A) *Didymella pinodes* isolate FT07005, (B) *Phoma medicaginis* var. *pinodella* isolate FT07007, (C) *Phoma koolunga* isolate DAR78535 and (D) *Phoma koolunga* isolate FT07006 after 3 and 7 d incubation on potato dextrose agar (PDA) amended with filtrate of each of the isolates and 3 and 7 d after transfer to normal PDA. Vertical bars represent standard errors of the means. Least significant differences for interaction between isolate × filtrate ($P < 0.001$); 3 d on amended PDA = 2.4, 7 d on amended PDA = 5.4, $P < 0.001$; not significant for unamended PDA.

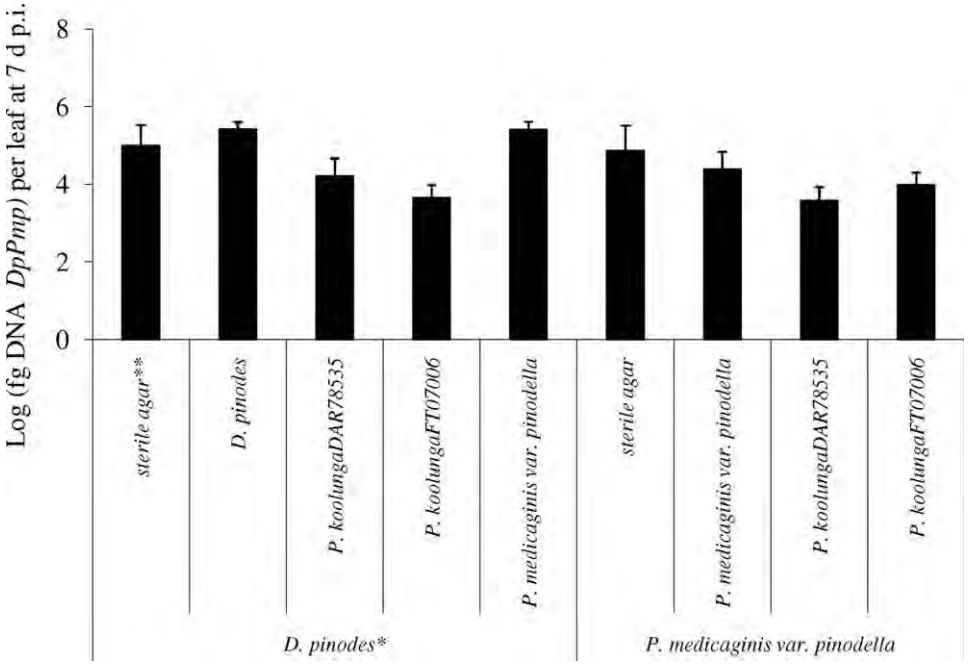

FIG. 2. Quantity of DNA of *Didymella pinodes* plus *Phoma medicaginis* var. *pinodella* (*DpPmp*) in intact plants of field pea cv. Kaspa pair co-inoculated with combinations of *Didymella pinodes* isolate FT07005, *Phoma medicaginis* var. *pinodella* isolate FT07007, *Phoma koolunga* isolate DAR78535, *Phoma koolunga* isolate FT07006 and sterile agar plug (control) at 7 d post inoculation (p.i.). Vertical bars represent standard errors of the means. Least significant difference ($P < 0.01$) = 1.12. * Denotes first of the co-inoculated isolates, ** denotes the other isolate of co-inoculated pair or sterile agar plug for controls.

isolates, including *D. pinodes*, while the diameter of *P. medicaginis* var. *pinodella* was reduced 11–13% (FIG. 1B) when grown on medium amended with filtrate from the *P. koolunga* isolates. The diameter of the *P. koolunga* isolates was not significantly different from the controls (FIG. 1C, D).

When transferred from filtrate-amended PDA to unamended PDA all cultures grew as well as the respective controls after 3 d incubation (FIG. 1). After 7 d on unamended PDA the diameter of *P. koolunga* DAR78535 was reduced by 17% in two instances in the first experiment (data not shown) while the diameters of *D. pinodes* and *P. medicaginis* var. *pinodella* were reduced respectively by 10% and 5% in two and three instances in the second experiment.

Comparison of lesion size and quantity of pathogen DNA on leaves of intact plants.—Inoculation with pairs of isolates did not influence the size of lesions on leaves of intact field pea plants, but quantity of pathogen DNA at 7 d p.i. varied with the composition of the pairs. For lesion size at 3 and 7 d p.i. the interaction terms and main effects for co-inoculant were not significant, indicating that inoculation of leaves with pairs of isolates did not influence the diameter of lesion due to either co-inoculant. At 3 d p.i. the mean lesion diameter due to *D. pinodes* (5.4 mm) averaged across co-inoculants was significantly (L.S.D. = 1.4, $P < 0.001$) larger than that caused by the other three fungal isolates. Inoculation with *P. medicaginis* var. *pinodella* resulted in the smallest lesions (mean 1.6 mm diam), significantly ($P < 0.001$) less than those due to *P. koolunga* FT07006 (mean 3.6 mm diam) but statistically equivalent to *P. koolunga* DAR78535 (mean 2.9 mm diam). At 7 d p.i. the mean lesion diameter, averaged across co-inoculants, caused by *D. pinodes* (9.3 mm) was still significantly (L.S.D. = 0.7, $P < 0.001$) larger than that caused by the other three isolates. The lesions caused by *P. koolunga* DAR78535 (mean 5.4 mm diam) were significantly smaller than those caused by the other three isolates, while those caused by *P. medicaginis* var. *pinodella* (mean 7.0 mm diam) and *P. koolunga* FT07006 (mean 6.5 mm diam) were similar.

The quantity of DNA per leaf of each of the co-inoculated pathogens at 3 d p.i. did not differ from the amount of DNA when co-inoculated with sterile agar (data not shown). However at 7 d p.i. the amount of DNA of *D. pinodes* and of *P. medicaginis* var. *pinodella* per leaf was significantly ($P < 0.01$) reduced when co-inoculated with *P. koolunga* FT07006 or DAR78545 (FIG. 2). The quantity of DNA of *P. koolunga* was not influenced by co-inoculation with any of the fungal isolates (data not shown).

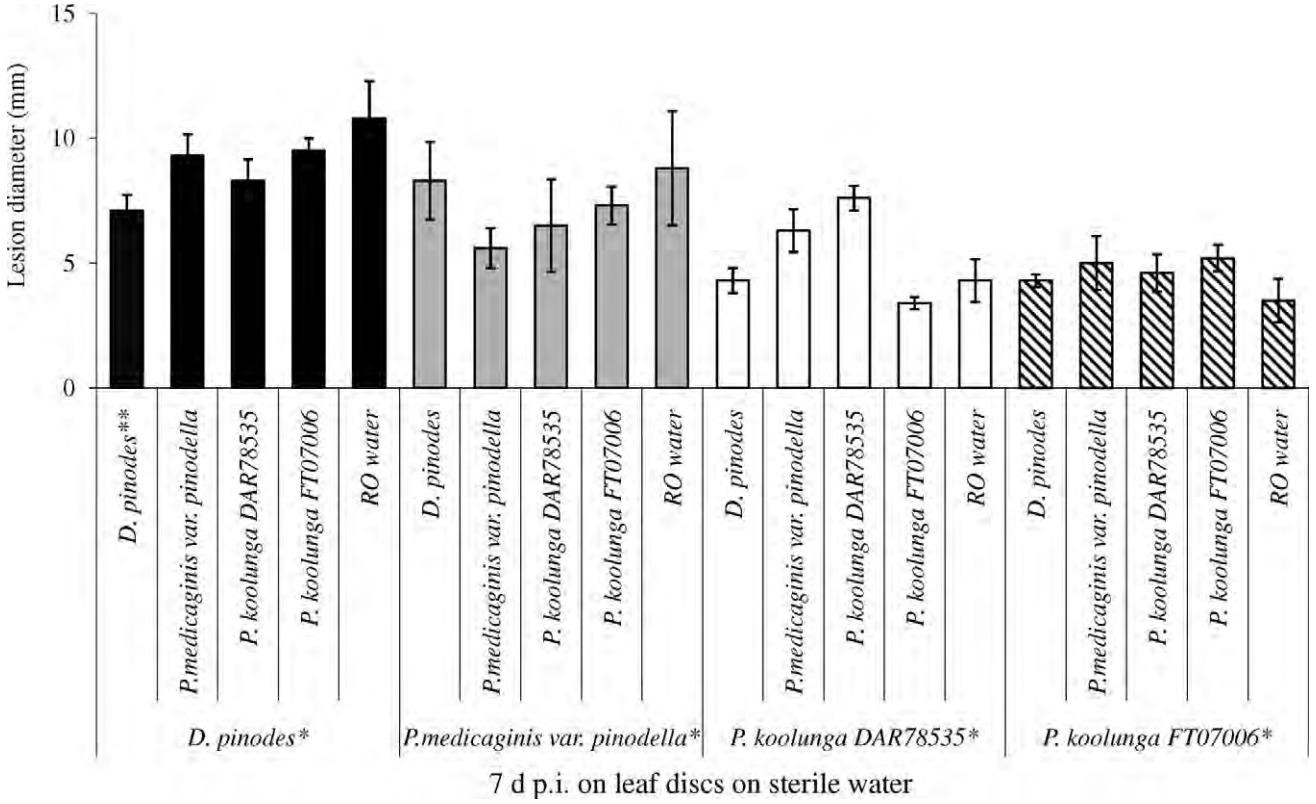

FIG. 3. Diameter of lesions caused by *Didymella pinodes* isolate FT07005, *Phoma medicaginis* var. *pinodella* isolate FT07007, or *Phoma koolunga* isolates DAR78535 and FT07006 when co-inoculated with each other or with sterile reverse osmosis (RO) water (control) at 7 d post inoculation (p.i.) on leaf disks of field pea cv. Kaspa floated on sterile RO water. Inoculum of paired isolates (or sterile water) was placed 5 mm apart. Vertical bars represent standard errors of means. Maximum least significant difference (for comparing between isolates co-inoculated with self) = 3.2; average least significant difference (for comparing between isolates co-inoculated with self and other combinations) = 2.5; minimum least significant difference (for comparing all other combinations) = 1.9; $P < 0.001$. *Denotes isolate that caused lesion measured, ** denotes co-inoculated isolate or sterile water for controls.

Comparison of lesion size and quantity of pathogen DNA on excised leaf disks.—Co-inoculation of the pathogen isolates on disks from field pea produced variable results depending on the substrate supporting the tissue. When disks were floated on sterile RO water the lesion size was influenced by the co-inoculated pathogen while quantity of pathogen DNA did not differ. However the co-inoculated pathogen had no influence on lesion size or on quantity of pathogen DNA when the disks were supported on water agar.

For disks on sterile RO water at 3 d p.i. the interaction terms and main effects for co-inoculant were not significant, indicating that inoculation of disks with pairs of isolates did not influence the lesion diameter due to either isolate at this assessment. The mean lesion diameter averaged across co-inoculants caused by *D. pinodes* (6.5 mm) was significantly (L.S.D = 0.5, $P < 0.001$) larger than the diameter of lesions caused by the other isolates (2.6–2.8 mm). However at 7 d p.i. the interaction term for co-inoculation was significant at $P < 0.001$ (FIG. 3). When co-inoculated with *P. koolunga* DAR78535 the diameter of the lesions caused by *D. pinodes* (8.3 mm) was significantly (L.S.D. min = 1.9. $P < 0.001$) reduced, in comparison with the control (*D. pinodes* co-inoculated with sterile RO water, 10.8 mm). A similar reduction in lesion diameter was observed when *P. medicaginis* var. *pinodella* (6.5 mm) was co-inoculated with *P. koolunga* DAR78535 compared to the control (8.8 mm). Lesions caused by *D. pinodes* and *P. medicaginis* var. *pinodella* were also significantly smaller than their respective controls when self-paired (7.1 and 5.6 mm respectively; L.S.D. ave = 2.5, $P < 0.001$). Lesions caused by *P. medicaginis* var. *pinodella* were smaller than those caused by *D. pinodes* when paired with the same co-inoculant, except when co-inoculated with *D. pinodes*. Conversely the diameter of lesions caused by *P. koolunga* DAR 78535 was significantly increased above that of controls on sterile RO water (4.3 mm) when self-paired (7.6 mm,

L.S.D av = 2.5) or when co-inoculated with *P. medicaginis* var. *pinodella* (6.3 mm, L.S.D. min = 1.9). However the diameter of lesions caused by *P. koolunga* DAR78535 was reduced significantly below that of the control when co-inoculated with *P. koolunga* FT07006 (2.4 mm, L.S.D. min = 1.9). The quantity of DNA in these disks was not influenced by the co-inoculated isolate at either assessment. When the experiment was repeated with water agar to support the disks the co-inoculated isolate had no influence on lesion size or on quantity of pathogen DNA.

DISCUSSION

This study represents the first report of interactions among *D. pinodes*, *P. koolunga* and *P. medicaginis* var. *pinodella*, causal agents of ascochyta blight on field pea in southern Australia. Antagonism, including self-antagonism, was detected among these species, leading to reduction in lesion size and quantity of pathogen DNA. The slower growing species, *P. koolunga*, was not self-antagonistic and in a few instances the effect of co-inoculation was additive or synergistic. *P. koolunga* grew more slowly on PDA than either *D. pinodes* or *P. medicaginis* var. *pinodella* and consistently caused smaller lesions than *D. pinodes* on leaves of field pea plants in controlled conditions. The latter reaction had been noted in Davidson et al. (2009a). It might be expected from these results that *P. koolunga* would be a minor contributor to the ascochyta blight complex on field pea, but the pathogen was isolated from diseased field peas at a similar frequently as *D. pinodes* in South Australia (Davidson et al. 2011). Also *P. koolunga* caused almost as much disease on field pea as did *D. pinodes* in controlled experiments (Davidson et al. 2009). In a preliminary experiment co-inoculation onto PDA resulted in zones of inhibition between colonies of *P. koolunga* and the faster growing species including between self-paired colonies of a single *P. koolunga* isolate. Colonies of *D. pinodes* and *P. medicaginis* var. *pinodella* were smaller on PDA amended with filtrates from *P. koolunga* than corresponding controls, and likewise colonies of *D. pinodes* were smaller when exposed to filtrate from cultures of *P. medicaginis* var. *pinodella* and *D. pinodes*. These observations suggested that these fungi might secrete compounds that are inhibitory, even to self.

Antagonism between *D. pinodes* and *P. medicaginis* var. *pinodella* was noted by Le May et al. (2009), such that smaller lesions and fewer pycnidia were produced when the two species were co-inoculated simultaneously on the same leaf on pea plants. They observed no reduction when the pathogens were inoculated on different parts of the host plant. However Le May et al. (2009) did not pair inocula of a single species on the same leaf and they did not examine interactions in vitro, so no comparison can be made with the current finding that *D. pinodes* or *P. medicaginis* var. *pinodella* can be self-inhibitory when co-inoculated. Furthermore Le May et al. (2009) inoculated plants with a suspension of conidia while mycelial plugs served as inoculum in the present study. Further studies are required to investigate the effect of the different inoculation methods on the interactions between these species and the consequences for lesion size, quantity of fungal DNA and sporulation. In addition using small numbers of conidia of each species, as would occur during rain, in simultaneous and sequential inoculations may reveal more about the antagonistic and synergistic interactions demonstrated by these two studies and identify the dominant colonizer.

In the current study the inhibitory effect of filtrate of *P. koolunga* did not persist when the cultures were transferred to unamended PDA, indicating that the filtrates were fungistatic rather than fungicidal. The filtrates from *D. pinodes* and *P. medicaginis* var. *pinodella* had no obvious influence on the growth of the *P. koolunga* isolates. Although some variability in colony size was observed after transfer to unamended PDA, this is likely to reflect experimental variability. Initially mycelial plugs of *P. koolunga*, *D. pinodes* and *P. medicaginis* var. *pinodella* were placed separately onto cellophane membranes overlaid on PDA to examine diffusible antibiosis (Dennis and Webster 1971b, Etebarian et al. 2000). However all three pathogens digested the cellophane, indicating the cellulolytic nature of these fungi.

P. koolunga also appeared to antagonize the other two pathogens in leaves, in that the quantity of DNA of *D. pinodes* and *P. medicaginis* var. *pinodella* in leaves on intact plants was reduced when co-inoculated with *P. koolunga* even when the size of the lesion caused by each pathogen was unaffected. Conversely on leaf disks floating on sterile RO water, whereas there were no differences in quantity of DNA, the size of lesions due to *D. pinodes* and *P. medicaginis* var. *pinodella* was reduced by co-inoculation with *P. koolunga* and lesions due to *D. pinodes* also were reduced by self-pairing. *P. koolunga* demonstrated a synergistic or additive relationship with *P. medicaginis* var. *pinodella* or when self-paired, in that lesion size increased on disks simultaneously inoculated with the conidial suspensions. Le May et al. (2009) detected synergism between *D. pinodes* and *P. medicaginis* var. *pinodella* on intact plants only when the two species were inoculated several days apart. However no interac-

tions between the pathogens were noted in the current study when the disks were supported by water agar instead of sterile RO water.

The anomaly in the response on leaves of intact plants and disks may reflect the influence of environmental conditions on the plant-pathogen relationship. When moisture is freely available *D. pinodes* has been reported to break down the phytoalexin, pisatin, letting lesions expand rapidly (Heath and Wood 1971). It is possible that in the current study the flotation of disks on water provided an environment in which antifungal metabolites could be denatured more rapidly by *D. pinodes*, confounding results. On the other hand antifungal metabolites may have leached from the leaf into the water. As noted above Le May et al. (2009) found that when *D. pinodes* and *P. medicaginis* var. *pinodella* were simultaneously inoculated onto field pea there was a reduction in lesion size if they were applied to the same leaf but not if applied to different parts of the same plant. This suggests that the interaction occurs within the leaf instead of being systemic, so disks may be considered suitable substitutes for intact plants. Disks have been used successfully in screening germplasm of field pea for resistance to ascochyta blight pathogens (Richardson et al. 2009). It is possible that wounding the plant material by excising disks might have contributed to the anomalies in results, but because entire detached leaves deteriorated rapidly in water they were not suitable for this study. Other inconsistencies may have arisen from the different inoculation methods.

Further investigation is required to understand the potential role of fungistatic metabolites in the relationship among *P. koolunga*, *D. pinodes* and *P. medicaginis* var. *pinodella* and whether such metabolites help *P. koolunga* compete with the other pathogens for space and nutrients on the field pea plant. Plant pathogenic fungi are reported to produce a wide array of antimicrobial toxins with effects such as reduced mycelial growth and conidial production (Duffy et al. 2003). There is some evidence in the literature that fungi that grow slowly and infect later are more frequently antagonistic to competing fungi (Gloer 1995), and this may apply to *P. koolunga*. Some pathogens also can degrade toxins produced by other pathogens, which could be a mechanism to gain a competitive advantage (Duffy et al. 2003). *D. pinodes* is known to produce phytotoxic compounds (Evidente et al. 1998, Shiraishi et al. 1991), but there are no reports of production of metabolites toxic to fungi by any of the pathogens investigated here. However production of antifungal metabolites by *Trichoderma* species (Ghisalberti and Sivasithamparam 1991), *Colletotrichum graminicola* (Wicklow et al. 2009) and by endophytic fungi (Yu et al. 2009) is well documented. Phytoalexins also are reported to have inhibitory activity against secondary colonists (Duffy et al. 2003), which may further confound the antagonistic relationship among these pathogens.

The competitive nature of *P. koolunga* indicates that this pathogen is likely to remain an important component of the ascochyta blight complex on field pea in South Australia. *Phoma koolunga* appears to be common in South Australia but less so in other pea-growing regions of Australia, whereas *D. pinodes* and *P. medicaginis* var. *pinodella* are widespread. *Phoma koolunga* and *D. pinodes* were found to be equally responsible for ascochyta blight symptoms in naturally infected field pea trials, whereas *P. medicaginis* var. *pinodella* had a minor role in the disease complex. (Davidson et al. 2011). Further studies are warranted to identify the fungistatic compound(s) produced by *P. koolunga*, if it is produced in planta, and the role this might play in the competitive relationship among the causal pathogens of ascochyta blight complex on field pea.

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Chapter 6.

Relationship between ascochyta blight on field pea (*Pisum sativum*) and spore release patterns of *Didymella pinodes* and other causal agents of ascochyta blight.

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Relationship between ascochyta blight on field pea (*Pisum sativum*) and spore release patterns of *Didymella pinodes* and other causal agents of ascochyta blight

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Ascochyta blight of field pea, caused by *Didymella pinodes, Phoma medicaginis* var. *pinodella, Phoma koolunga* and *Didymella pisi,* is controlled through manipulating sowing dates to avoid ascospores of *D. pinodes*, field selection and foliar fungicides. This study investigated the relationship between number of ascospores of *D. pinodes* at sowing and disease intensity at crop maturity. Field pea stubble infested with ascochyta blight from one site was exposed to ambient conditions at two sites, repeated two years. Three batches of stubble with varying degrees of infection were exposed at one site, repeated three years. Every two weeks, stubble samples were retrieved, wetted and placed in a wind tunnel and up to 2500 ascospores g^{-1} h^{-1} were released. Secondary inoculum, monitored using seedling field peas as trap plants in canopies arising from three sowing dates and external to field pea canopies, was greatest in early sown crops. A model was developed to calculate the effective number of ascospores using predictions from G1 Blackspot Manager (Salam *et al.*, 2011b), distance from infested stubble (Salam *et al.*, 2011a) and winter rainfall. Maximum disease intensity was predicted based on the calculated number of effective ascospores, soilborne inoculum and spring rainfall over two seasons. Predictions were validated in the third season

with data from field trials and commercial crops. A threshold amount of ascospores of *D. pinodes*, 294 g^{-1} stubble h^{-1}, was identified, above which disease did not increase. Below this threshold there was a linear relationship between ascospore number and maximum disease intensity.

Keywords: Blackspot Manager, *Mycosphaerella pinodes*

Introduction

Ascochyta blight is the most common disease of field pea in Australia (Davidson & Ramsey, 2000; Bretag *et al*., 2006), often causing 25% yield loss and sometimes up to 75% yield loss in individual crops (Bretag *et al*., 1995a; McDonald & Peck 2009; McMurray *et al*., 2011). This disease, which has a worldwide distribution, is caused by the fungal pathogens *Didymella pinodes, Phoma medicaginis* var. *pinodella* and *D. pisi*. *Phoma koolunga* was recently identified as another component of the disease complex in South Australia (Davidson *et al*., 2009). *D. pinodes* is considered the major pathogen in this complex, and it produces airborne ascospores as primary and secondary inoculum that are spread long distance by wind (Bretag *et al*., 2006). *D. pisi* is rarely associated with ascochyta blight in southern Australia. Inoculum of the two *Phoma* species consists of rain-splashed conidia (Davidson *et al*., 2009; Punithalingham and Gibson, 1976), although the perfect stage of *P. medicaginis* var. *pinodella* has been reported in laboratory conditions (Bowen *et al*., 1997).

Limited genetic resistance to ascochyta blight has been identified in field pea germplasm (Bretag *et al*., 2006) and disease control depends on agronomic practices such as delayed sowing and strategic application of foliar fungicides to minimise infection. However, foliar fungicides are not always cost effective in the low rainfall environments of southern Australia where field pea yield is often less than 2 t ha^{-1}, while in the high and medium rainfall environments the requirement for economic benefit restricts fungicide use to no more than two applications per crop (McMurray *et al*., 2011).

The major control strategy for ascochyta blight of field pea in Australia until recently has been to delay sowing crops until 4–6 weeks after the first autumn rains, to minimise infection from airborne ascospores of *D. pinodes* (Bretag *et al*., 2000; Davidson & Ramsey 2000). However, delayed sowing in southern Australia often leads to yield loss due to heat and moisture stress during flowering and grain filling stages (McDonald & Peck, 2009; McMurray *et al*., 2011). In response to recent weather patterns comprising less rainfall and shorter growing seasons, optimum sowing dates were revised to 3 weeks after the first autumn rains in medium and medium–high rainfall regions, and to within 1 week in low rainfall regions (McMurray *et al.,* 2011).

Daily rainfall and temperature influence the timing of ascospore release from infested field pea stubble, leading to seasonal and regional variation in ascochyta blight risk. Consequently, a forecasting system for predicting release of ascospores of *D. pinodes*, G1 Blackspot Manager, was developed to identify sowing dates that minimised the risk of ascochyta blight without delaying sowing longer than necessary. Growers were advised to delay sowing until at least 50% of the ascospores had been released and fallen on bare soil (Salam *et al.,* 2011b).

G1 Blackspot Manager predicts the fraction of the ascospores available in a given season that are released on individual days but the total number of ascospores available as inoculum in that season can vary widely. Hence the rule of sowing after 50% of ascospores have been released relates to a wide range of potential inoculum. In addition, the absolute number of ascospores is influenced by proximity to infested stubble (Salam *et al*., 2011a) and it is likely that the number of ascospores also varies with the severity of disease on the stubble. The effect of amount of ascospores available as inoculum on disease development has not been established.

The ability of the ascospores to infect field pea is affected by humidity during the infection process (Schoeny *et al*., 2007) which, in turn, will affect disease. In addition, disease

severity at the end of the growing season is influenced by secondary inoculum cycles of conidia and ascospores within the crop. Conidia, which develop from pycnidia within ascochyta blight lesions, are dispersed short distances by rain (Schoeny *et al*. 2008) but, under Australian conditions, these are considered of minor importance (Bretag *et al*., 2006). However, pseudothecia of *D. pinodes* develop on infected, senescent leaves and on infected stems during crop maturation, releasing ascospores which spread quickly throughout the crop. Subsequent rainfall events promote infection by these ascospores, further increasing disease (Bretag *et al.,* 2006; Roger & Tivoli, 1996). Consequently, there appears to be no direct relationship between the number of ascospores released from infested stubble and disease severity at the end of the growing season, although G1 Blackspot Manager identifies disease risk from the pattern of ascospore release from stubble.

Other sources of inoculum can also be important in the early establishment of ascochyta blight in field pea crops e.g. soilborne inoculum which is able to survive for several years in the absence of the host (Bretag *et al*., 2006; Davidson *et al*., 2011; Wallen *et al*., 1967, Wallen and Jeun, 1968). While the pathogens are commonly detected on seed, seed-borne inoculum is not considered a source of inoculum for ascochyta blight epidemics (Bretag *et al*. 1995b; Moussart *et al*. 1998).

The aim of this study was to (i) establish the relationship between disease development and numbers of ascospores of *D. pinodes* released, the secondary inoculum of all the causal pathogens produced within the crop canopy, and rainfall; and (ii) to develop a disease predictor, for ascochyta blight of field pea and validate it with independent field data.

Materials and Methods

Ascochyta blight in field pea disease management trials

Ascochyta blight was assessed in naturally infected field pea disease management trials described in detail by McMurray *et al.* (2011) in three regions which differed in annual

rainfall and length of growing season. These trials were conducted in 2007, 2008 and 2009 in part to assess fungicide efficacy and the effect of time of sowing on disease and yield. The experimental sites were located in three areas of South Australia as follows (i) a medium – high rainfall region (mean annual rainfall 464 mm) represented by Kingsford Research Station (34.5ºS 138.8ºE), 50 km north of Adelaide, in 2007 and 2009 and the nearby Turretfield Research Station (34.6ºS 138.8ºE) in 2008; (ii) medium rainfall (mean annual rainfall 429 mm) and short growing season represented by Hart, 140 km north of Adelaide (33.8ºS 138.4ºE), in 2007, 2008 and 2009; and (iii) a low rainfall (mean annual rainfall 325 mm) and short growing season represented by Minnipa (32.9ºS 135.2ºE) approximately 600 km north west of Adelaide, in 2007, 2008 and 2009. The trials at the medium – high rainfall sites and the medium rainfall site each had three times of sowing, with the first sowing date as soon as practicable after the first autumn rains (Early Sown) and subsequent sowing dates 3 weeks (Medium Sown) and 6 weeks (Late Sown) later. The trials at the low rainfall site consisted of the two earlier times of sowing, as the latest sowing date was impractical for agronomic reasons.

In order to estimate the amount of soilborne inoculum present at each site, 500 g of soil were collected per site, prior to sowing as described in Davidson *et al*. (2011) and subjected to DNA tests for *D. pinodes* plus *P. medicaginis* var. *pinodella* and *P. koolunga* as described in Davidson *et al.* (2011). The relationship between quantity of soilborne DNA and disease intensity on field pea plants at end of winter (designated DiseaseAugSoil) was inferred from data in this previous study using Equation 1 below.

DiseaseAugSoil (number of girdled internodes) = (5.0584 * log DNA of pathogens (pg g^{-1} soil) – 6.0153)/27.1

Equation 1

Disease intensity was measured by sampling six plants of field pea cv. Kaspa collected at random from untreated buffer plots, established at each time of sowing, every 2 weeks for the trials at Hart, Kingsford and Turretfield, and every 4 weeks for the trials at Minnipa, from seedling stage in June to crop maturity in October. The plants were assessed for the number of internodes on the main stem with 100% area diseased (termed 'girdled internodes'). Internodes with partial infection were assessed for proportion of surface area diseased and the fraction was added to the total number of girdled internodes. Data for the six plants assessed per sowing treatment were averaged. Microscopic examination of representative lesions on stems and leaves of each plant was conducted to determine the presence or absence of pseudothecia containing ascospores of *D. pinodes*, and pycnidia containing conidia of *D. pinodes*, *P. medicaginis* var. *pinodella* and *P. koolunga*.

Daily weather data (rainfall, maximum and minimum temperature) for the three sites for each season were accessed through Silo patchpoint (SILO 2010). The Rosedale data point represented Kingsford and Turretfield (being 7 km east of Kingsford and 2 km south west of Turretfield), the Blyth data point represented Hart (11 km south of the site) and the Minnipa data point was on-site. Rainfall at each site x sowing date x year was summed from sowing date to end of July (designated RainWinter) and summed for August and September (designated RainAS).

Disease intensity (number of girdled internodes) at the end of August (i.e. end of vegetative phase) was regressed against RainWinter and the number of additional internodes that became girdled during crop maturation (i.e. September and October) was regressed against RainAS, using Genstat 14. These relationships were accommodated in the maximum disease estimator described below.

Estimation of airborne primary inoculum from infested stubble

The number of ascospores of *D. pinodes* released from infested field pea stubble was assessed in different seasons and in different regions using stubble with varying degrees of infection. Each year from 2006 to 2009, naturally infected field pea crops and research trials with ascochyta blight were selected following random monitoring. Disease was assessed on standing crops prior to harvesting as described above for 20 plants selected at random, and stubble was collected from these crops immediately after harvest (Table 1). In December 2006 and November 2007 stubble was collected from Kingsford. In December 2007, and November 2008 and 2009 three lots of field pea stubble, each with different degrees of disease intensity (Table 1), were collected from commercial field pea crops within a 10-km radius of Hart. Disease intensity on the Hart stubble was categorised as low, moderate or high based on disease observations within each season; 4, 7 and 15 girdled internodes respectively on stubble collected in December 2007 and 4, 8 and 13 girdled internodes respectively on stubble collected in November 2008. The stubble collected from the Hart region in November 2009 followed a severe ascochyta blight epidemic and the low disease category comprised 9 girdled internodes, moderate 12 girdled internodes and high 19 girdled internodes. Stubble was stored in dry conditions for 1 to 7 weeks until processed. Segments of approximately 12 cm of the stems with ascochyta blight lesions were placed into nylon mesh bags (20 x 20 cm with pore size 1 mm^2, 20 pieces per bag). Ascochyta blight lesions completely covered more than seven girdled internodes of the stem pieces. The stubble was incubated on the soil surface at either Kingsford or Hart, whichever was closest to the stubble collection site (Table 1). Steel mesh (7.5 x 7.5 cm grid size) was laid over the nylon mesh bags to prevent disturbance by wind and animals were excluded with a 1-m high steel mesh enclosure. In 2007 and 2008 the stubble collected from Kingsford was also incubated at the Waite Campus, Urrbrae (34.9°S 138.6°E). An automatic weather station (AWS) (Measurement Engineering Australia) was placed at each site to record daily rainfall and daily average temperature.

Missing data were substituted by data from the nearest Bureau of Meteorology site (http://www.bom.gov.au/climate/data).

Every 2 weeks a bag of stubble representing each category of disease intensity was collected from each incubation site (Table 1). The bags of stubble were sent to the Northam laboratory of the Department of Agriculture and Food Western Australia (DAFWA), where the number of mature ascospores was assessed. Each stubble sample was wetted for 5 min and placed in a wind tunnel for 1 h, such that *D. pinodes* ascospores released from the stubble were captured on sticky tape (1 x 30 mm) mounted on rotor rods. The number of ascospores on the sticky tape was counted using a light microscope at 400x magnification (Galloway & MacLeod, 2003; Salam *et al*., 2011b) and data presented as ascospores g^{-1} stubble h^{-1}. The number of ascospores captured every 2 weeks from the different batches of stubble incubated at Hart was compared within a season using correlation analysis in Genstat 14.

Monitoring of secondary inoculum

The timing of release and relative amounts of secondary inoculum (conidia and ascospores) of ascochyta blight pathogens were monitored for field pea canopies with different sowing dates and regressed against rainfall and disease intensity in the field pea canopy. The relative number of spores (airborne, soilborne and/or splash dispersed combined) at Kingsford and Turretfield Research Stations in the canopy for each sowing date over three seasons (2007 to 2009) was monitored indirectly by counting the number of lesions on trap plants of field pea seedlings as described by Roger and Tivoli (1996) and Schoeny *et al.* (2007). Trap plants consisted of a tray containing 3-week-old field pea seedlings, cv. Parafield, 12 per tray, that were raised in the greenhouse before being placed in the field. Trap plant placement is detailed in Table 2. All trap plants within the field pea canopies were placed in control plots which received no fungicide applications. After 7 days of exposure the trap plants were returned to the greenhouse and placed in plastic trays covered with lids. Water was added to

the trays to a depth of 2 cm to provide high humidity and the temperature was maintained between 18 and 25ºC. After 4 days the trap plants were placed on an open bench so that humidity decreased, the plants were incubated for a further 3 days and the total number of lesions on each plant in the tray was counted. The mean number of lesions per plant was calculated for each tray. Lesions on trap plants external to the field pea canopy from May to end of July were assumed to be caused by primary inoculum from infested stubble and from soilborne inoculum. Lesions on trap plants within the field pea canopy were assumed to be caused by both primary inoculum and secondary inoculum produced in the lesions on the infected plants. The relative amounts of secondary inoculum from each canopy from May to end of July were estimated by subtracting the number of lesions on trap plants outside the trials from the number of lesions on traps within the trials. After July, when airborne primary inoculum was depleted, the relative amounts of secondary inoculum per canopy were estimated as the total number of lesions on the traps inside the pea canopies; soilborne inoculum was presumed to be equivalent across the three times of sowing in each trial.

The average number of lesions on the weekly trap plants adjacent to infested pea stubble in 2007 and 2008 was compared with the number of ascospores released from infested pea stubble over the same period, using Spearman's rank correlation in Genstat 14. The significance of the correlation coefficient was determined in this analysis using Student's t distribution with $n - 2$ degrees of freedom at 5% probability.

Multiple linear regression in Genstat 14 was used to analyse the relationship between (i) the total weekly rainfall (mm) and the average number of lesions on the weekly trap plants in canopies of the disease management trials from 2007 to 2009, and (ii) disease intensity in the disease management trials as number of girdled internodes (averaged over the six plants) and the average number of lesions on the weekly trap plants. Data across years were tested for homogeneity before pooling. Lesion numbers and disease were square root-transformed for the regression analyses to standardise the residuals.

Maximum disease estimator

The experiments described above, as well as predictions from G1 Blackspot Manager (Salam *et al.*, 2011b) and data on distance from infested stubble (Salam *et al.*, 2011a), were used to develop a three-step model that estimated (i) the number of ascospores available as primary inoculum, designated 'effective number of ascospores', (ii) maximum disease intensity at the end of winter using the relationship between the calculated effective number of ascospores and rainfall recorded in the disease management trials described above and, (iii) the final maximum disease intensity at the end of the growing season as the sum of the disease at the end of winter plus disease following rainfall during crop maturation (August and September), using data collected from the disease management trials in 2007 and 2008 described above. This model was named the 'maximum disease estimator'.

The maximum disease estimator started with either 5000 or 10000 ascospores per region [SporesInit] based on the total number of ascospores captured from the stubble at Hart and Kingsford each year and the observation that numbers larger than 10000 saturated the model. SporesInit numbers were reduced according to the following ratios: (a) % ascospores remaining on infested stubble at sowing for each site x time of sowing as predicted by G1 Blackspot Manager (Salam *et al*., 2011b) [%Spores]; (b) distance from known infested field pea stubble around trial sites [Distance] (Salam *et al*., 2011a); (c) relationship between cumulative winter rainfall [RainWinter] from sowing to end of winter (August) and disease intensity as observed in the disease management trials. A rainfall lower limit was set for no disease and an upper threshold of rainfall was set for maximum disease. The effective number of ascospores was calculated using Equation 2.

Effective number of ascospores (ascospores g^{-1} stubble h^{-1}) = SporesInit (∑ascospores g^{-1} stubble h^{-1}) X %Spores X Distance (m) X RainWinter (mm).

Equation 2.

The effective number of ascospores calculated in Equation 2 was regressed against the observed disease intensity at end of winter (August) less the amount of disease attributed to soilborne inoculum (Equation 1) in all trials and sowing dates to generate the relationship in Equation 3. One data point was omitted from the regression since the observed disease at this point was much less than the observed disease for similar numbers of ascospores in other year x site combinations. The amount of disease from soilborne inoculum (DiseaseAugSoil) was added to Maximum Disease (Aug) in Equation 3.

Maximum Disease (Aug) (girdled internodes) = 0.0354 X effective number of ascospores (ascospores g^{-1} stubble h^{-1}) + 0.0246 + DiseaseAugSoil (girdled internodes).

Equation 3.

A maximum disease intensity was set for the end of winter based on observations in the disease management trials. Stepwise correlation analysis was performed in Microsoft Office Excel 2007 between the effective number of ascospores calculated in Equation 1 and the observed disease intensity (number of girdled internodes) at the end of August to identify when the number of girdled internodes reached a maximum, after which any additional ascospores had minimal influence on disease. The maximum value for this parameter was included in the final disease model.

Finally, the relationship between rainfall in August and September [RainAS] and disease intensity during crop maturation was added to the disease calculated in Equation 3.

Final maximum disease (girdled internodes) = Disease (Aug) (girdled internodes) + 0.0475 X RainAS (mm) - 0.9798.

Equation 4.

Model parameters are shown in Table 3. Final maximum disease intensity predicted for all the sowing dates and sites in the 2007 and 2008 trials was plotted against the effective numbers of ascospores calculated in Equation 2, and the final maximum disease intensity predicted for the sowing dates x sites for the medium-high and medium rainfall regions in 2007 and 2008 was linearly regressed [PredictedRegression] against the percentage of ascospores present at sowing calculated from G1 Blackspot Manager (Salam *et al.,* 2011b).

Survey of commercial field pea crops and validation of maximum disease estimator

Data from the 2009 field trials described above and from a survey of commercial field pea crops in 2009 described below were used to validate the maximum disease estimator. Each year from 2007 to 2009, all field pea crops within a 10-km radius of Hart were identified and mapped. Approximate sowing dates were calculated in winter from the mean number of internodes on 20 plants selected arbitrarily in the crops. This information was used to group crops into sowing categories similar to the sowing dates in the field trials described above; Early (late April to early May), Medium (Mid – late May) and Late (early June onward). No data were available on crop rotation or soilborne inoculum. Crops representative of each sowing group were selected for assessment of ascochyta blight in late September or October. Selection within each sowing group was based on proximity to infested field pea stubble, such that crops on or adjacent to, within 500 m of, or more than 500 m from infested stubble were represented. Twenty plants were selected in a W transect across the field (Davidson *et al*., 2001), one every 50 paces. Plants were assessed for the growth stage (vegetative, flowering, early pods, mature pods), total number of internodes, and number of internodes girdled by ascochyta blight. The effect of sowing period on disease intensity was analysed in 2007 using

two–sample t-tests in Genstat 14; no data were collected for proximity to field pea stubble in 2007. In 2008 and 2009 the effect of sowing period and proximity to field pea stubble on disease intensity was analysed by unbalanced analysis of variance in Genstat 14 using crops as replicates.

To validate the maximum disease estimate, the observed disease intensity in the 2009 field trials and in the 2009 commercial crops was compared with maximum disease intensity predicted by the maximum disease estimator using Lin's concordance correlation coefficient. This analysis assesses the linear relationship between the two measurements and the degree to which the pairs fall on the 45° line through the origin (Lin 1989). Where multiple observations had the same maximum disease prediction, only the maximum observation was included in the correlation, since only maximum disease observations were used to generate the relationship in Equation 3 above.

Linear regression, in Genstat 14, was performed between observed disease intensity at the end of the season and the percentage of ascospores present at sowing calculated by G1 Blackspot Manager (Salam *et al*., 2011b). The regression slope was compared with the slope for the Predicted Regression described above, using t-tests at $\alpha = 0.05$.

Results

Ascochyta blight in field pea disease management trials

No disease was observed when cumulative rainfall from sowing (April to May) until the end of July was less than 50 mm. Disease intensity at the end of winter (August) reached a maximum of 11 girdled internodes when the cumulative rainfall, from sowing until the end of July, was more than 100 mm. There was a significant linear regression ($r^2 = 0.621$, P = <0.001) for cumulative rainfall over this period between 50 and 100 mm rainfall and disease intensity at the end of August (Fig. 1). This result was used to set lower (< 50 mm) and upper (> 100 mm) limits for winter rainfall categories in the maximum disease estimator (Table 3).

There was also a significant regression ($r^2 = 0.597$, P = <0.001) between the increase in disease in spring (September and October) and the rainfall during August and September.

The combined quantity of DNA of the pathogens (*D. pinodes*, *P. medicaginis* var. *pinodella* and *P. koolunga*) prior to sowing the field pea trials was zero in the Minnipa trials, 153-171 pg g^{-1} of soil in 2007 trials, 1024-1510 pg g^{-1} of soil in 2008 and 23-490 pg g^{-1} of soil in the 2009 trials at Hart, Kingsford and Turretfield. The disease intensity at the end of August attributed to the soilborne inoculum was estimated using Equation 1 to be less than 0.37 internodes per plant in all trials.

Estimation of airborne primary inoculum from infested stubble

The total number of ascospores released from infested stubble and captured in the wind tunnel in one season was between 3962 and 9986 at Kingsford and the Waite Campus respectively (Table 1). In 2007 and 2008, ascospores were first detected from stubble retrieved in January and detection ceased in June at the Waite Campus in both years and in late July or August at Kingsford in 2007 and 2008, respectively. The patterns of spore release at Kingsford and the Waite Campus were significantly correlated in both seasons (2007, r = 0.74, P < 0.001; 2008, r = 0.59, P < 0.01). There was a peak in ascospores for stubble samples in April-May in both years, associated with rainfall above 25 mm in a 2 week period. An additional peak in February 2007 from stubble incubated at Kingsford in 2007 coincided with 40.2 mm of rainfall in the 2 week incubation period. At the Waite Campus, over the same period, rainfall was less than 14 mm, and few ascospores (0-661 g^{-1} h^{-1}) were recorded at this site in the summer months of both years. Another variation occurred in the 2 weeks preceding and following 2 April 2007, when more spores were captured from stubble incubated at Kingsford than at the Waite Campus; this was associated with four additional rain days at the former site. Ascospore release from May to July during 2008 peaked slightly later at Kingsford than

at the Waite Campus; Kingsford had fewer rain days, and or less rainfall, than the Waite Campus in all but one of the 2 week incubation periods.

The total number of ascospores captured in the wind tunnel in each season at Hart was similar to that of Kingsford and the Waite Campus for stubble with low and moderate disease intensity (as described in Table 1), between 4205 and 11830, except in 2008 when the stubble with moderate disease released 53320 ascospores over the season. The stubble with most disease released up to 159059 ascospores in one season (Table 1). At Hart, ascospores were trapped from stubble samples collected from January until mid-September, mid-July and late June in 2008, 2009 and 2010, respectively, and the maximum numbers were recorded during the months of April and May, coinciding with rainfall of more than 20 mm in a 2 week period.

The numbers of ascospores released from the three lots of field pea stubble incubated each year at Hart were significantly ($P<0.05$) correlated within each season (Table 4), except for the stubble from plants with high disease intensity compared to stubble with moderate disease intensity collected in 2009. Although release patterns were similar for each stubble sample, the disease intensity on the stubble at the time of harvest affected the number of ascospores detected. In 2008 and 2009 more ascospores were released from the stubble with high disease intensity than from the stubble samples with low or moderate disease intensity. The largest number released from a stubble sample in 2008 and 2009 was 26205 and 91695 ascospore g^{-1} h^{-1}, respectively. However, in 2010 more ascospores (22866 ascospores g^{-1} h^{-1}) were obtained from the stubble with moderate disease intensity (12 girdled internodes) rather than from stubble with high disease intensity (19 girdled internodes). The stubble incubated in 2010 was the most severely diseased of all the material used, however the largest number of spores captured in the wind tunnel occurred in 2009 from stubble with disease comprising 13 girdled internodes.

Monitoring of secondary inoculum

The average number of ascochyta blight lesions on the field pea seedlings in trays placed near infested field pea stubble or outside but close to field pea trials at the beginning of the growing season (May) varied from 1281 per seedling in 2007 to below 30 per seedling in 2008 (Fig. 2). In June and July in both years, the average number of lesions was below 40 per seedling and remained at this low level for the rest of the season. Conversely, mean numbers of lesions on trap plants placed within the field pea canopy increased as the season progressed, reaching a maximum in September each year, with peaks of 177, 805 and 341 lesions per seedling in 2007, 2008 and 2009, respectively (Fig. 2). In 2008, one earlier peak of 548 lesions per seedling occurred in August and in 2009 there were four earlier peaks, ranging from 133 to 211 lesions per seedling, from July to August. Associated with these peaks, rainfall was greater in June and July 2009 than in 2007 and 2008. There was a total of 130 mm from the start of the growing season to early July 2009, while only 95 mm fell by early August 2008 and a similar amount by September 2007. Pseudothecia containing ascospores of *D. pinodes* were detected on field pea plants in the trials during these peak periods, as were pycnidia containing conidia of *D. pinodes*, *P. koolunga* and *P. medicaginis* var. *pinodella*. Conidia were also observed outside these peak periods (data not shown).

The correlation between numbers of ascospores trapped from infested field pea stubble and lesions on trap plants in 2008 was significant ($r = 0.86$, $P = 0.006$) for traps placed next to infested field pea stubble and significant ($r = 0.71$, $P = 0.053$) for traps placed next to the field pea disease management trial. Numbers of ascospores and numbers of lesions on trap plants showed similar patterns in 2007 (Fig. 3).

Within a season, earlier sowing resulted in more lesions on trap plants, presumed to arise from secondary inoculum (Fig. 2). In 2008, trap plants placed in the first time of sowing treatment had a total of 2396 lesions for the season, those in the second time of sowing treatment had a total of 1238 lesions and those in the third time of sowing treatment, 295

lesions. In 2009 the total number of lesions on trap plants placed in the first, second and third times of sowing treatments was 2117, 877 and 606, respectively.

The number of lesions (square root) on the trap plants within the field pea canopy, analysed using all data from 2007 to 2009, increased ($P<0.001$, $r^2 = 0.4$) with increasing disease intensity (number of girdled internodes) in the trial and with weekly rainfall ($P<0.002$, $r^2 = 0.4$). The interaction between rainfall and plant disease was not significant. Few lesions were observed on trap plants when trial plants had an average of less than 1 girdled internode, although where rainfall of 33.4 mm was recorded large numbers of lesions developed on trap plants placed in the canopy even though the average disease intensity on the plants in the trial was less than 2 girdled internodes. Numerous lesions were typically observed on trap plants when total weekly rainfall was 10 mm or more and also when rainfall was less (6.2 mm) if the trial plants had an average of 4 or more girdled internodes (Fig. 4).

Maximum disease estimator

There was a significant linear relationship between the effective number of ascospores and number of girdled internodes at the end of winter (August) (Equation 2 in Materials and Methods); and the stepwise correlation analysis identified a maximum disease intensity at the end of August of 11 girdled internodes at 294 g^{-1} h^{-1} effective ascospores, after which disease did not increase.

Disease increase after winter was correlated with rainfall in August and September (Fig. 1). Maximum disease intensity at the end of the growing season was estimated using the model for each sowing date in each disease management trial and plotted against the calculated effective number of ascospores (Fig. 5). Disease intensity increased linearly (Disease at end of growing season [observed] = 1.567 + 0.0386 X effective number of ascospores; $r^2 = 0.802$, $P < 0.001$) to a maximum of 20 girdled internodes when effective numbers of ascospores were 294 g^{-1} h^{-1}, after which the disease did not increase. The value of

0.37 girdled internodes at the end of August attributed to soilborne inoculum was calculated to increase up to 5 girdled internodes by the end of the season due to the additional effect of spring rainfall. The predicted maximum disease intensity at the end of the season had a linear relationship with percentage of ascospores at sowing for the combined data from Hart, Kingsford and Turretfield sites in 2007 and 2008 ($r^2 = 0.687$, $P < 0.001$) (Fig. 6). At these sites 50% of ascospores remaining at sowing resulted in a predicted disease intensity of 10 or fewer girdled internodes. At the low rainfall site, Minnipa, the estimated disease in 2007 and 2008 remained at zero girdled internodes when 75% of ascospores remained at sowing, since low rainfall reduced effective ascospores to less than 8% of the potential number.

Survey of commercial field pea crops and validation of maximum disease estimator

In 2007, 2008 and 2009, 52, 45 and 41, respectively, commercial field pea crops were mapped within a 10-km radius of Hart. Sowing dates were evenly spread in all three seasons; 32.6% were in the Early sown category, 35.6% were in the Medium sown category and 32.7% were sown Late. The majority of the crops were in close vicinity to infested field pea stubble from the previous season; 49.4% were either adjacent to or planted into field pea stubble, 29.9% were no more than 500 m from field pea stubble, and only 20.6% of crops were more than 500 m from field pea stubble. All crops were affected by ascochyta blight and disease was assessed in 18, 15 and 22 crops in 2007-09, respectively.

In each consecutive year of the study, disease significantly increased with earlier sowing ($P \leq 0.01$) (Table 5). The intensity of ascochyta blight ranged from 0.4 to 14.8 (average 5.4) girdled internodes in 2007, and from 1.8 to 12.7 girdled internodes (average 5.9) in 2008. In 2009 the minimum disease intensity was 8.3 and the maximum was 20.2 girdled internodes (average 13.2). In 2008 proximity to infested stubble significantly ($P<0.01$) increased ascochyta blight at each sowing period. Disease was lowest in crops sown in the

mid or late period which were not adjacent to infested stubble (Table 5b). In 2009 disease was lowest in crops sown in the late period not adjacent to infested field pea stubble (Table 5c).

There was a significant correlation ($r = 0.8436$, $P < 0.001$) and concordance ($C_b = 0.9339$) between observed maximum disease intensity and predicted maximum disease intensity for the 2009 field trials and the survey of commercial field pea crops in the same year. There was also a significant linear relationship ($r^2 = 0.294$, $P < 0.001$) between observed disease at the end of the season in 2009 and percentage of ascospores present at sowing predicted by G1 Blackspot Manager (Fig. 6). There was no significant difference between the slopes of the linear regressions between % ascospores present at sowing, and observed or predicted disease.

Discussion

A threshold number of ascospores of *D. pinodes* above which severity of ascochyta blight on field pea did not increase was identified. Below this threshold there was a linear relationship between numbers of ascospores and maximum disease intensity. This finding verified the assumption made in the G1 Blackspot Manager model (Salam *et al*., 2011b) that the timing of peak release, identified through the percentage of the total ascospores available at a given time, and sowing date were the primary factors influencing ascochyta blight disease in field pea in southern Australia. At the peak the numbers of ascospores released from the stubble exceeded the threshold for maximum disease intensity and crops should be sown after this event to limit development of epidemics. Not only were early sown canopies exposed to more primary inoculum, they also produced ascospores before the later sown canopies and in greater numbers. Winter and spring rainfall was positively correlated with disease intensity, and disease was not observed when winter rainfall was less that 50 mm, an important finding for low rainfall regions.

The effective number of ascospores calculated by the maximum disease estimator varied with the initial numbers on the stubble, the percentage of spores remaining at sowing (according to G1 Blackspot Manager), rainfall and with distance from infested stubble. Disease intensity at the end of winter, when primary inoculum was no longer present, reached a maximum of 11 internodes girdled, irrespective of the effective number of ascospores. This was supported by the data used for validation, where an estimated 3500 effective spores did not result in more disease than 294 effective spores, whereas from 0 to 294 effective spores, there was a linear relationship with maximum disease. A similar relationship between ascospores discharged from infested stubble and disease has been reported for blackleg (phoma stem canker) of canola (oilseed rape) (Wherrett *et al.,* 2004). The results from the current study confirmed that timing of ascospore release is more important than the absolute numbers of spores. Crop growth stage and rainfall at the time of maximum ascospore release are likely to be determining factors in the establishment of disease on the crops during autumn and winter. Schoeny *et al.* (2003, 2007) also related ascochyta blight severity in field pea to rainfall during disease onset and crop growth.

The maximum disease estimator revealed that when 50% or less of ascospores were present at sowing, the disease intensity at crop maturity was 10 internodes or less (approximately 30% of entire stem) girdled with ascochyta blight. This is in agreement with the 20 - 40% of stem infected calculated by Salam *et al*. (2011b) from Western Australian data. Salam *et al.* (2011c) estimated the yield loss associated with this disease score to be 20% or less and similar figures were observed in the disease management trials in South Australia described by McMurray *et al.* (2011). In the low rainfall region of Minnipa, a higher percentage of spores can be present at sowing without increasing the risk of disease. This information is especially important for farmers in these dry regions where early sowing is essential to allow maximum yield potential.

The total number of ascospores released from infested field pea stubble per season varied with site and season but the factors that influence seasonal variation are not known. The total number was not directly related to disease on the stubble incubated in these experiments. It was anticipated that most ascospores would be obtained from the 2009 stubble incubated in 2010, on which disease intensity was greatest, but this did not occur. It is possible that when numerous crops of ascospores are released within the canopy as secondary inoculum, as occurred in 2009, the pathogen in the subsequent stubble is depleted and produces fewer ascospores as primary inoculum in the following autumn. Field pea residues are known to develop successive crops of mature pseudothecia, initially prolific, exhausting the inoculum by 50 weeks (Carter 1963; McDonald and Peck 2009).

Ascospore numbers did vary with disease intensity on stubble within a season, showing that the more diseased the crop the greater the potential to release ascospores from the subsequent stubble. Irrespective of amount of disease, the pattern of release remained the same within a site and season. In 2010 more ascospores were obtained from the stubble with moderate disease than from the most diseased stubble, although the apparent anomaly may have been due to experimental error.

Although airborne ascospores of *D. pinodes* are considered the primary inoculum during establishment of field pea crops, soilborne inoculum has also been associated with disease (Davidson *et al.* 2011), but distinguishing between the different sources of inoculum is difficult. Using the relationship identified by Davidson *et al*. (2011) to estimate the effect of soilborne inoculum in these trials indicated that a minor component of the inoculum in these trials came from the soil, and the majority from airborne ascospores. Nevertheless, when soilborne inoculum is more abundant than measured here, combined with high rainfall it has the potential to cause significant disease. As data on soilborne inoculum were not available in the survey of commercial crops, this aspect could not be considered in the validation process, which may have contributed to variability in the results.

Lesions on trap plants attributed to secondary inoculum increased during August or later as the crop matured, depending on rainfall and disease in the crop. This secondary inoculum results in an increase in ascochyta blight epidemics in late winter and spring (Bretag, 1991; Roger and Tivoli, 1996). Examination of the trap plant data identified the compounding effect of time of sowing on secondary inoculum. In the current study, the number of spores produced in-crop was greatly increased by early sowing where conditions were conducive for disease, and ascospores were produced much earlier in early-sown crops than in crops sown later, leading to more ascochyta blight in spring. Late-sown crops are exposed to relatively few ascospores from the previous year's stubble and, subsequently, produce fewer in-crop spores, leading to little disease. These results support the practice of later sowing to control ascochyta blight in field pea, as was demonstrated in the field trials. Roger and Tivoli (1996) found that the number of conidia produced in-crop increased with disease. In the current study the greatest peaks of secondary inoculum and increase in disease occurred when pseudothecia containing ascospores were readily detected on the diseased plants, confirming that ascospores as secondary inoculum have an important role in an epidemic (Bretag, 1991). As ascospores are produced on senescent plant material, early intervention to control disease to prevent premature senescence at the base of the plant could be a strategy to minimise or limit the development of epidemics (Roger and Tivoli, 1996).

Production of secondary inoculum in ascochyta blight-affected field pea crops was also strongly linked to quantity and timing of rainfall, and the progressively earlier detection of lesions on trap plants placed within field pea canopies from 2007 to 2009 was linked to higher rainfall during the later growing seasons. Given that there was no interaction between disease intensity and rainfall in terms of the number of lesions detected on trap plants, rainfall was deemed the most suitable parameter for use in the predictive model to calculate the increase in disease during spring.

The survey of commercial field pea crops in the Hart district provided support for the previous observations on the influence of time of sowing and distance from infested pea stubble on disease, the latter identified by Salam *et al*. (2011a). Many growers appear to have ignored basic agronomic disease management strategies of distance from stubble and or delayed sowing. This may be due to constraints in field selection on the property and the yield risk associated with short dry seasons when sowing is delayed. In these circumstances, G1 Blackspot Manager allows growers to identify the disease risk linked to their agronomic decisions.

In conclusion, there was a threshold number of ascospores of *D. pinodes* from infested field pea stubble below which this primary inoculum was directly related to disease intensity at the end of winter. Disease did not increase above this threshold. The primary inoculum was also a determining factor in the amount of secondary inoculum produced in the crop, with rainfall leading to increased disease during spring. The influence of rainfall meant that the management of this disease was especially important in medium-high and medium rainfall areas. Research to identify reasons for the failure of industry to implement current recommendations for field selection and distance from infested stubble is warranted to improve the adoption of integrated disease management strategies aimed at minimising exposure to inoculum.

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Supporting information

Additional supporting information can be found in the online version of this article.

Figure S1 Numbers of ascospores (spores g^{-1} stubble h^{-1}) of *Didymella pinodes* captured in a wind tunnel from infested field pea stubble incubated at Kingsford, Turretfield or the Waite Campus in South Australia and retrieved at 2 week intervals from January to October in 2007 and January to December in 2008.

Figure S2 Rainfall (mm) at 2 week intervals at Kingsford and the Waite Campus in (a) 2007 and (b) 2008, and at Kingsford in (c) 2009.

Figure S3 Numbers of ascospores (spores g^{-1} stubble h^{-1}) of *Didymella pinodes* captured in a wind tunnel from infested field pea stubble, grouped in three categories of disease, incubated in the field at Hart in South Australia and retrieved at 2 week intervals from January to December in 2008, from February to August in 2009 and January to September in 2010.

Figure S4 Rainfall (mm) at 2 week intervals at Hart in (a) 2008, (b) 2009 and (c) 2010.

Table 1 Release of ascospores of *Didymella pinodes* from field pea stubble after incubation in the field. The total number of ascospores for the season and the number at peak release are presented; periodic data are presented in Figs 2 and 4

Site	Date stubble was collected	Date stubble returned to field for incubation	Spore trapping period[a]	Disease on stubble[b]	Total no. of ascospores per season (spores g^{-1} stubble)	No. of ascospores at peak release (spores g^{-1} h^{-1})
Kingsford	14 Dec 2006	21 Dec 2006	8 Jan – 1 Oct 2007	5	9986	2470
Waite Campus[c]	14 Dec 2006	21 Dec 2006	8 Jan – 1 Oct 2007	5	4492	2439
Kingsford	27 Nov 2007	19 Dec 2007	21 Jan – 22 Dec 2008	10	8096	2207
Waite Campus	27 Nov 2007	19 Dec 2007	21 Jan – 22 Dec 2008	10	3962	1997
Hart	15 Dec 2007	26 Dec 2007	21 Jan – 22 Dec 2008	4	4304	2384
Hart	15 Dec 2007	26 Dec 2007	21 Jan – 22 Dec 2008	7	4205	1642
Hart	15 Dec 2007	26 Dec 2007	21 Jan – 22 Dec 2008	15	71452	26205
Hart	11 Nov 2008	24 Nov 2008	10 Feb – 26 Aug 2009	4	11830	7555
Hart	11 Nov 2008	24 Nov 2008	10 Feb – 26 Aug 2009	8	53320	27653

Hart	11 Nov 2008	24 Nov 2008	10 Feb – 26 Aug 2009	13	159059	91695
Hart	28 Nov 2009	15 Jan 2010	29 Jan – 1 Sep 2010	9	9812	3307
Hart	28 Nov 2009	15 Jan 2010	29 Jan – 1 Sep 2010	12	51414	22866
Hart	28 Nov 2009	15 Jan 2010	29 Jan – 1 Sep 2010	19	8246	4027

[a]Stubble was sampled every 2 weeks.

[b]Disease was assessed as the number of stem nodes girdled by ascochyta blight lesions, averaged for 20 plants selected at random prior to harvest.

[c]Field pea stubble incubated at the Waite Campus was collected from Kingsford.

Table 2 Placement of field pea seedling trap plants[a] from 2007 to 2009

Year	Site	Position of trap	Trial sowing date	Date first trap placed in field	Date last trap placed in field
2007	Kingsford	10 m from infested field pea stubble and 300 m from field pea trial[b]	-[c]	24 Apr 2007	31 Oct 2007
2007	Kingsford	In trial, first time of sowing	10 May 2007	6 Jun 2007	31 Oct 2007
2008	Kingsford	10 m from infested field pea stubble	-	20 May 2008	5 Nov 2008
2008	Turretfield	15 m from field pea trial	9 May 2008	4 Jun 2008	5 Nov 2008
2008	Turretfield	In trial, first time of sowing[d]	9 May 2008	4 Jun 2008	5 Nov 2008
2008	Turretfield	In trial, second time of sowing	30 May 2008	25 Jun 2008	5 Nov 2008
2008	Turretfield	In trial, third time of sowing	12 Jun 2008	9 Jul 2008	5 Nov 2008
2009	Kingsford	In trial, first time of sowing	11 May 2009	20 May 2009	21 Oct 2009
2009	Kingsford	In trial, second time of sowing	1 Jun 2009	10 Jun 2009	21 Oct 2009
2009	Kingsford	In trial, third time of sowing	19 Jun 2009	8 Jul 2009	21 Oct 2009

[a]Trays of field pea seedlings, 12 seedlings per tray, were grown in the greenhouse until 3 weeks old then exposed to field conditions for 1 week, after which they were returned to the greenhouse to allow ascochyta blight lesions to develop.

[b]All trials were within 1.5 km of ascochyta blight-infested field pea stubble.

[c]No sowing date is presented since the trap plants were external to the field pea canopy.

[d]The disease management trials (McMurray *et al.*, 2011) included three times of sowing; trays were placed in the canopy arising from the first time of sowing in 2007 and in all three times of sowing in 2008 and 2009.

Table 3 Parameters of maximum disease estimator model

Parameter		Parameter value	Model factor
Initial ascospore numbers [SporesInit]	Kingsford, Turretfield & Minnipa, Hart 2009	-	10,000
	Hart 2007 and 2008	-	5000
Distance from infested field pea stubble (m) [Distance]		0	1.0000
		10	0.361
		50	0.14
		100	0.10
		400	0.03
Cumulative rain from sowing to end of August [RainWinter]		≥ 100 mm	1.0
		50 - 100 mm	(50-(100-x mm)/50
		< 50 mm	0
Maximum effective number of ascospores		-	294
Maximum disease at end of winter (Aug) (girdled nodes)		-	12
Parameter a^a for Disease at end of winter (Aug)			0.0246
Parameter b^b for Disease at end of winter (Aug)			0.0354
Parameter a^c for Disease at end of growing season			0.0475

Parameter b[d] for Disease at end of growing season	-0.9798

[a]Regression parameter for Disease at end of August' and 'Effective numbers of ascospores'.

[b]Regression constant for 'Disease at end of August' and 'Effective numbers of ascospores'.

[c]Regression parameter for 'Increase in Disease after Winter' and 'Cumulative Rainfall August and September'.

[d]Regression constant for 'Increase in Disease after Winter' and 'Cumulative Rainfall August and September'.

Table 4 Correlation between numbers of ascospores of *Didymella pinodes* captured in a wind tunnel each fortnight from field pea stubble and disease on the stubble incubated in the field at Hart, South Australia for 2008, 2009 and 2010

Year	Stubble disease[a] compared in correlation	Correlation coefficient	P value
2008	15 nodes vs 7 nodes	0.6247	P<0.001
2008	15 nodes vs 4 nodes	0.677	P<0.001
2008	7 nodes vs 4 nodes	0.4414	P<0.05
2009	13 nodes vs 8 nodes	0.9025	P<0.001
2009	13 nodes vs 4 nodes	0.975	P<0.001
2009	8 nodes vs 4 nodes	0.8008	P<0.01
2010	19 nodes vs 12 nodes	0.1334	Not Significant
2010	19 nodes vs 9 nodes	0.8186	P<0.001
2010	12 nodes vs 9 nodes	0.8627	P<0.001

[a]15, 13 and 19 nodes represent high disease; 7, 8 and 12 nodes represent moderate disease; 4, 4 and 9 nodes represent low disease.

Table 5 Mean intensity of ascochyta blight (number of girdled internodes) on 20 plants per crop sown in three sowing periods, either on or adjacent to, or not adjacent to infested field pea stubble in a 10-km radius of Hart, South Australia in 2007. Entries for 2007 are means of observations, entries for 2008 and 2009 are predictions from regression model generated by unbalanced analysis of variance

	Sowing period		
	Early (Early May)	Mid (Mid-late May)	Late (early June onward)
(a) 2007	12.4a[a] (3)[b]	4.9b (4)	3.7b (11)
(b) 2008			
Adjacent to or on field pea stubble	8.6a (3)	6.4b (3)	5.7bc (2)
Not adjacent to field pea stubble	4.7de (2)	5.2cd (3)	4.0e (2)
Maximum Least Significant Difference = 1.00; Average Least Significant Difference = 0.93; Minimum Least Significant Difference = 0.82.			
(a) 2009			
Adjacent or on field pea stubble	17.3a (3)	12.2c (4)	11.3c (3)
Not adjacent to field pea stubble	14.0b (4)	14.7b (5)	9.4d (3)
Maximum Least Significant Difference = 1.08; Average Least Significant Difference = 1.02; Minimum Least Significant Difference = 0.93.			

[a]Numbers followed by the same letter are not significantly different at P <0.001.

[b]Number of crops per category is in parentheses.

Figure captions

Figure 1 Linear regression between cumulative rainfall from sowing (May to June) to end of July and ascochyta blight, measured as number of girdled internodes, at the end of winter (August) on field pea plants in field trials in South Australia 2007 and 2008. Ascochyta blight (number of girdled internodes) = 0.1058 * Cumulative rainfall (mm) - 5.15; $r^2 = 0.62$; $P < 0.001$.

Figure 2 Number of lesions on trap plant seedlings, averaged for 12 field pea plants, placed for one week inside the canopy (solid line) or adjacent to (dotted line) field pea disease management trials in Kingsford or Turretfield, South Australia (see Table 2) (a) 2007 at Kingsford; (b) canopy arising from three times of sowing (TOS) at Turretfield in 2008 and (c) canopy arising from three TOS at Kingsford in 2009.

Figure 3 Number of ascospores of *Didymella pinodes* released from infested field pea stubble incubated at Kingsford, South Australia and the mean number of ascochyta blight lesions counted on trap plants; (a) in 2007 when trap plants were placed external to the adjacent field pea disease management trial, and

(b) in 2008 when trap plants were placed adjacent to infested pea stubble at Kingsford or adjacent to the field pea disease management trial at Turretfield, South Australia.

Figure 4 Square root of mean number of lesions on trap plants placed in field pea disease management trials at Kingsford in 2007 and 2009, and Turretfield in 2009 (McMurray *et al*., 2011) at weekly intervals compared with weekly rainfall (mm) and ascochyta blight (number of girdled internodes) in the disease management trials.

Figure 5 The relationship between the effective numbers of ascospores at sowing calculated by the maximum disease estimator and ascochyta blight (number of girdled internodes) at the end of the growing season (i) predicted disease in disease management trials at Kingsford, Turretfield, Hart and Minnipa, 2007 and 2008, (ii) observed disease in field trials at

Kingsford, Hart and Minnipa in 2009 and (iii) observed disease in commercial field pea crops in Hart district in 2009.

Figure 6 The linear relationship between the % of ascospores remaining at sowing (from G1 Blackspot Manager (Salam *et al*., 2011b)) and disease at the end of the growing season (i) in disease management trials at Kingsford, Turretfield and Hart in 2007 and 2008 predicted by maximum disease estimator (ascochyta blight (girdled internodes)) = 0.1179* % ascospores at sowing + 3.586, $r^2 = 0.687$, $P < 0.001$); and (ii) in 2009 field trials at Kingsford, Hart and Minnipa 2009 and in field pea crops in Hart district (10 km radius) in 2009 (ascochyta blight (girdled internodes)) = 0.1415 * % ascospores remaining at sowing + 0.83, $r^2 = 0.7294$, $P < 0.001$).

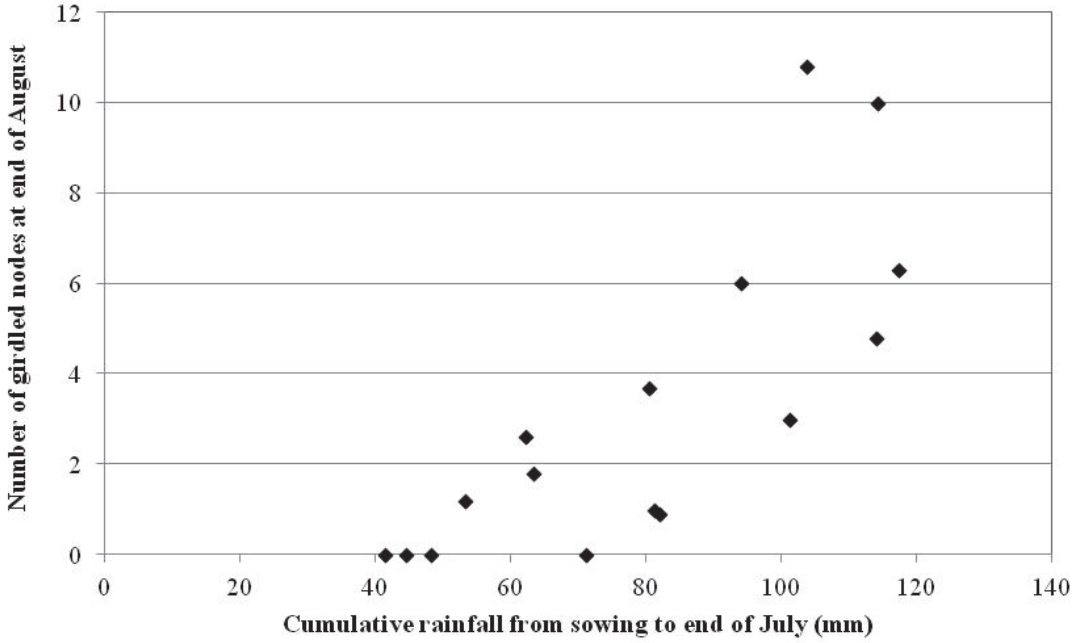

Number of girdled nodes at end of August
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
Cumulative rainfall from sowing to end of July (mm)

Figure 1

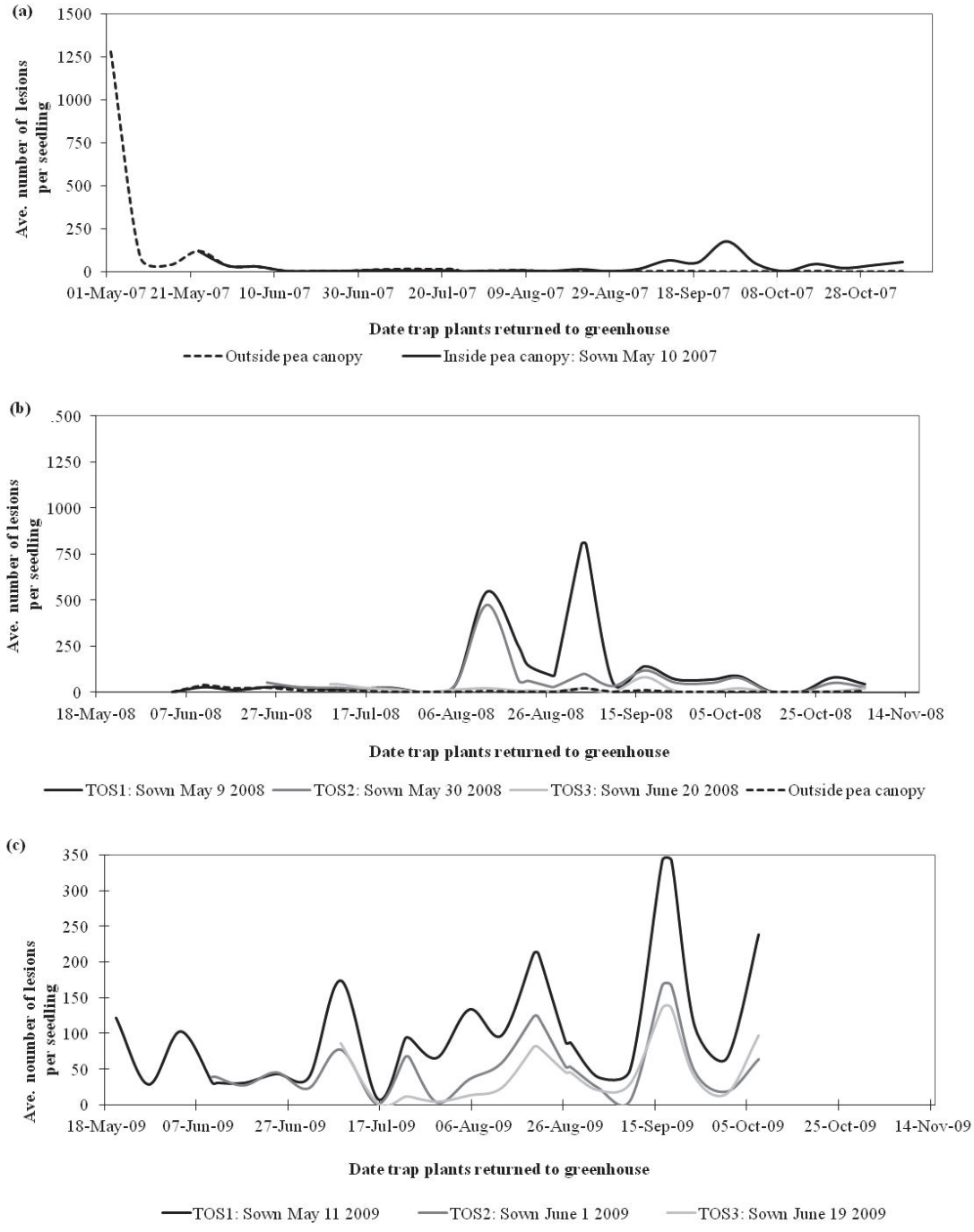

(a)
Ave. number of lesions per seedling
1500
1250
1000
750
500
250
0
01-May-07 21-May-07 10-Jun-07 30-Jun-07 20-Jul-07 09-Aug-07 29-Aug-07 18-Sep-07 08-Oct-07 28-Oct-07
Date trap plants returned to greenhouse
Outside pea canopy
Inside pea canopy: Sown May 10 2007
(b)
Ave. number of lesions per seedling
1250
1000
750
500
250
0
18-May-08 07-Jun-08 27-Jun-08 17-Jul-08 06-Aug-08 26-Aug-08 15-Sep-08 05-Oct-08 25-Oct-08 14-Nov-08
Date trap plants returned to greenhouse
TOS1: Sown May 9 2008
TOS2: Sown May 30 2008
TOS3: Sown June 20 2008
Outside pea canopy
(c)
Ave. noumber of lesions per seedling
350
300
250
200
150
100
50
0
18-May-09 07-Jun-09 27-Jun-09 17-Jul-09 06-Aug-09 26-Aug-09 15-Sep-09 05-Oct-09 25-Oct-09 14-Nov-09
Date trap plants returned to greenhouse
TOS1: Sown May 11 2009
TOS2: Sown June 1 2009
TOS3: Sown June 19 2009

Figure 2

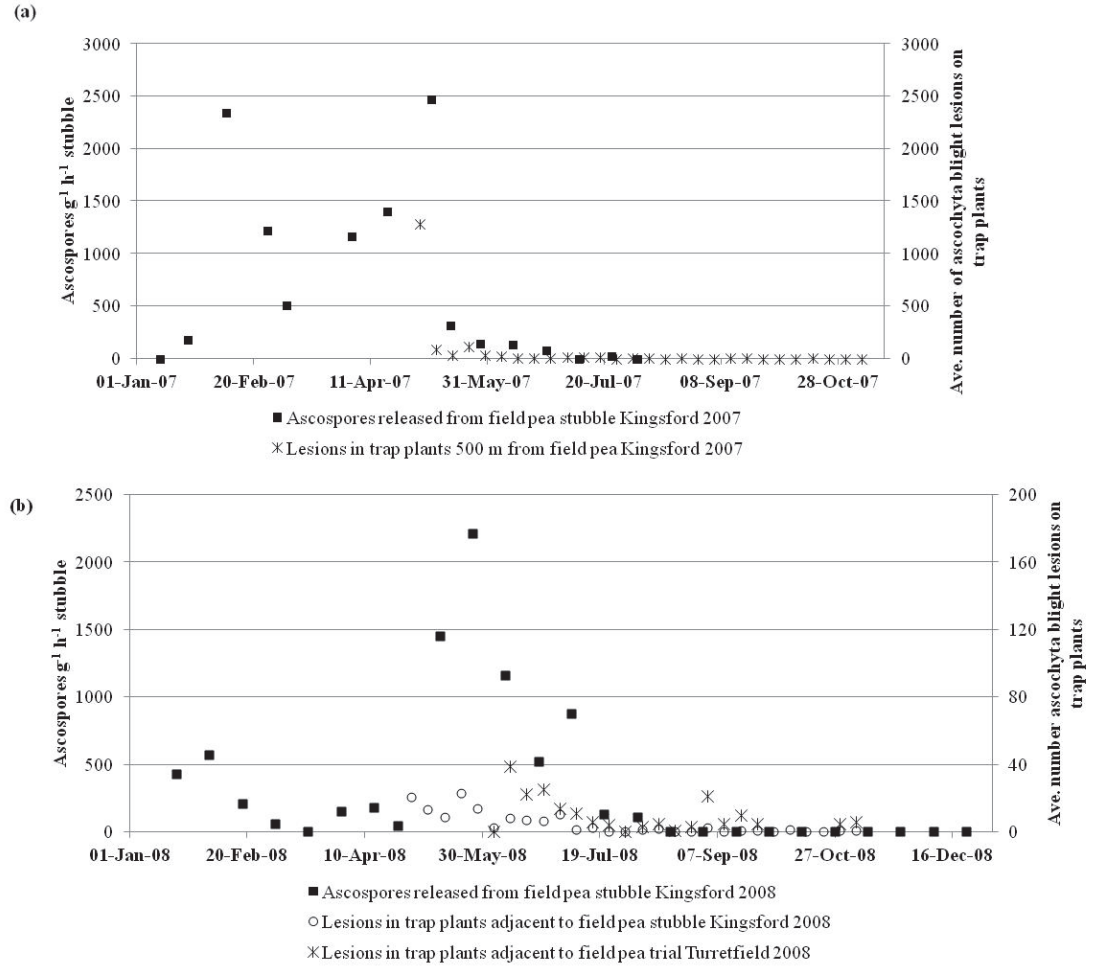

(a)
Ascospores g-1 h-1 stubble
Ave. number of ascochyta blight lesions on trap plants
3000
2500
2000
1500
1000
500
0
01-Jan-07
20-Feb-07
11-Apr-07
31-May-07
20-Jul-07
08-Sep-07
28-Oct-07
Ascospores released from field pea stubble Kingsford 2007
Lesions in trap plants 500 m from field pea Kingsford 2007
(b)
Ascospores g-1 h-1 stubble
Ave. number ascochyta blight lesions on trap plants
2500
2000
1500
1000
500
0
200
160
120
80
40
0
01-Jan-08
20-Feb-08
10-Apr-08
30-May-08
19-Jul-08
07-Sep-08
27-Oct-08
16-Dec-08
Ascospores released from field pea stubble Kingsford 2008
Lesions in trap plants adjacent to field pea stubble Kingsford 2008
Lesions in trap plants adjacent to field pea trial Turretfield 2008

Figure 3

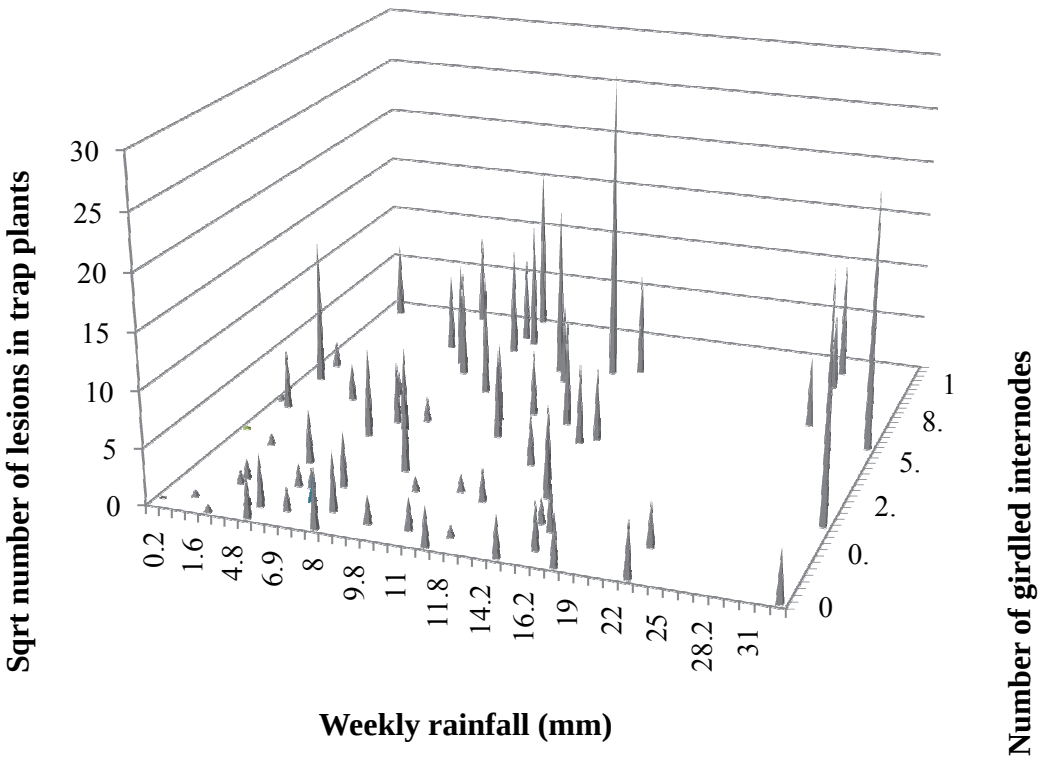

Sqrt number of lesions in trap plants
30
25
20
15
10
5
0
0.2
1.6
4.8
6.9
8
9.8
11
11.8
14.2
16.2
19
22
25
28.2
31
Weekly rainfall (mm)
0
0.
2.
5.
8.
1
Number of girdled internodes

Figure 4

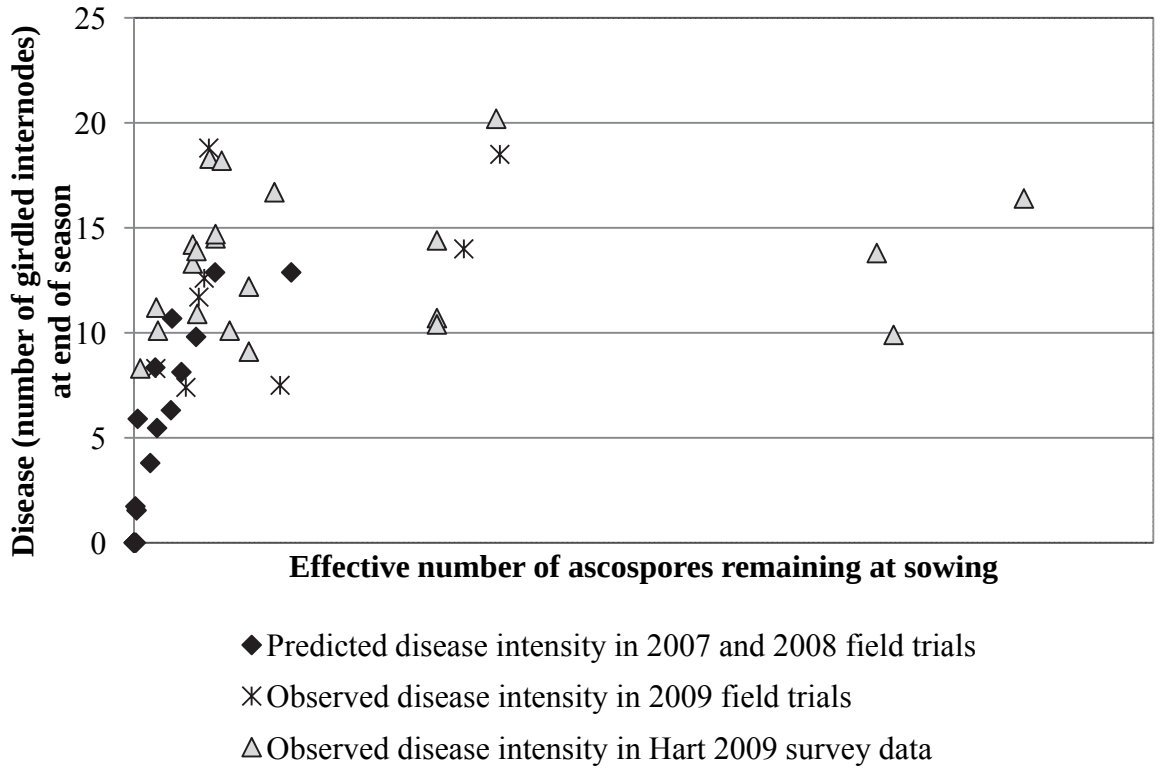

25
20
15
10
5
0
Disease (number of girdled internodes) at end of season
Effective number of ascospores remaining at sowing
Predicted disease intensity in 2007 and 2008 field trials
Observed disease intensity in 2009 field trials
Observed disease intensity in Hart 2009 survey data

Figure 5

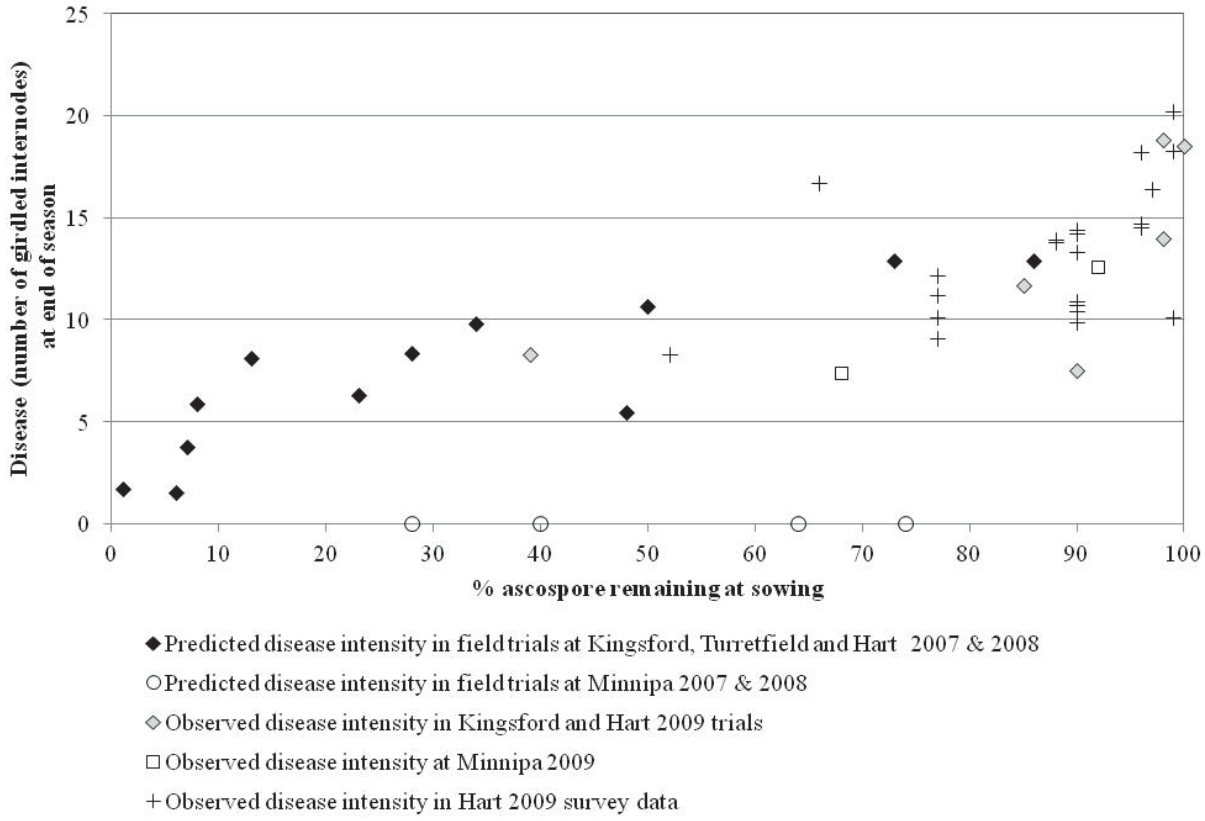

Disease (number of girdled internodes) at end of season
% ascospore remaining at sowing
0
5
10
15
20
25
0
10
20
30
40
50
60
70
80
90
100
Predicted disease intensity in field trials at Kingsford, Turretfield and Hart 2007 & 2008
Predicted disease intensity in field trials at Minnipa 2007 & 2008
Observed disease intensity in Kingsford and Hart 2009 trials
Observed disease intensity at Minnipa 2009
Observed disease intensity in Hart 2009 survey data

Figure 6

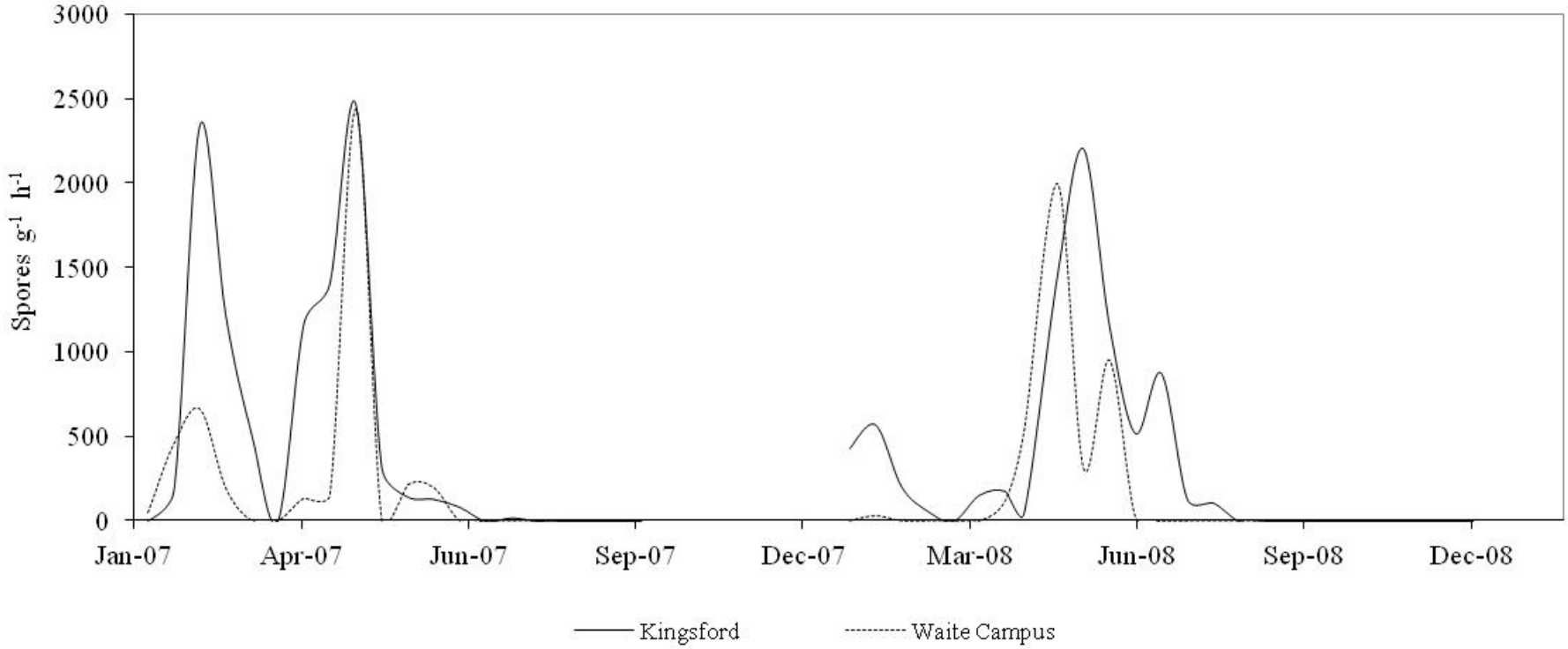
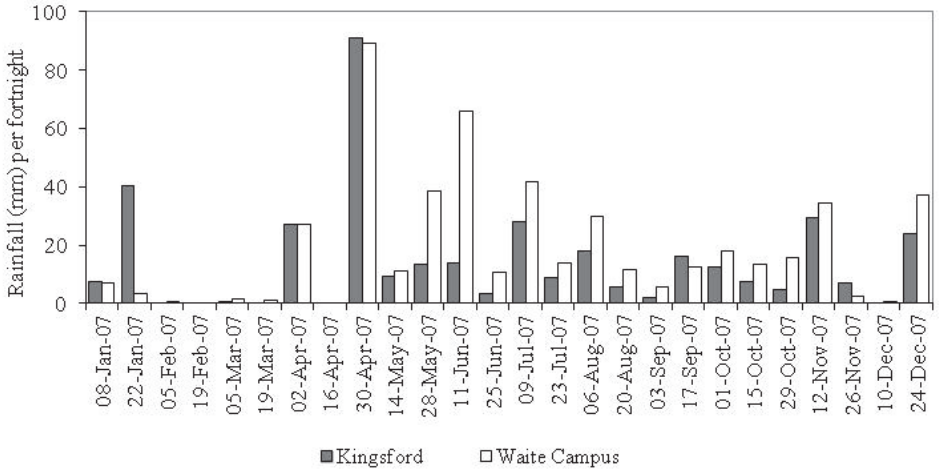
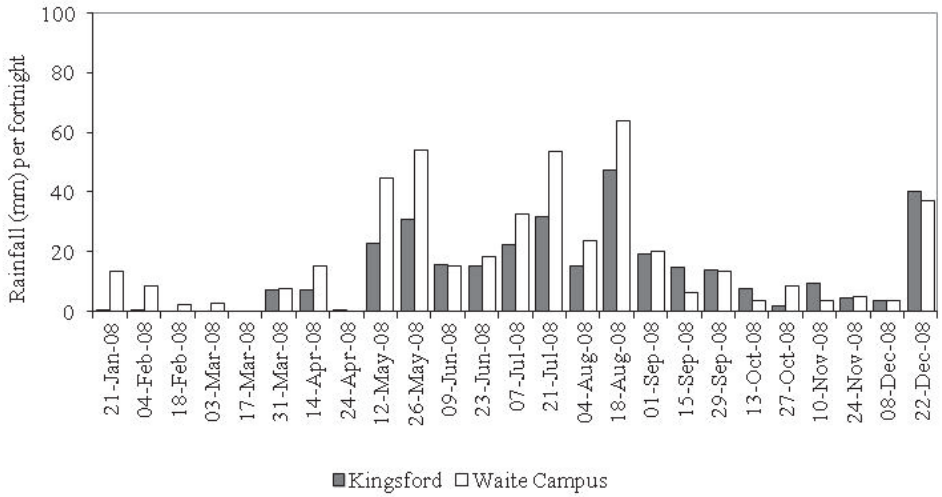

3000
2500
2000
1500
1000
500
0
Spores g-1 h-1
Jan-07
Apr-07
Jun-07
Sep-07
Dec-07
Mar-08
Jun-08
Sep-08
Dec-08
Kingsford
Waite Campus

Figure S1

(a)



(b)



(c)

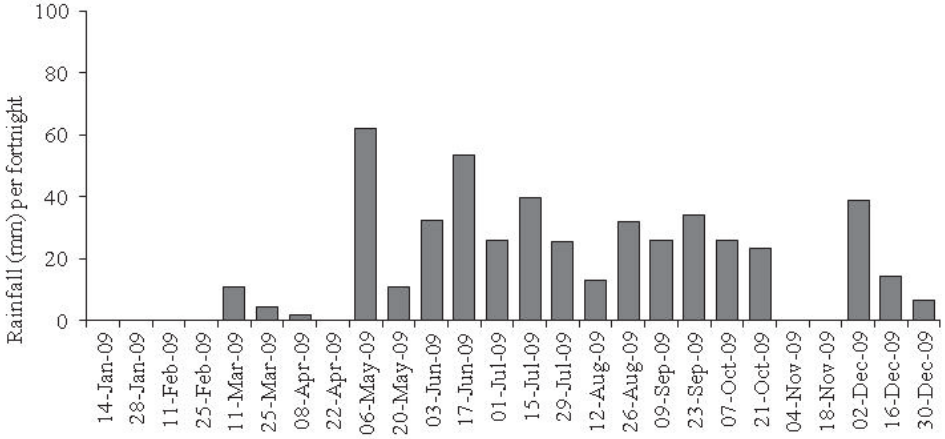


Figure S2

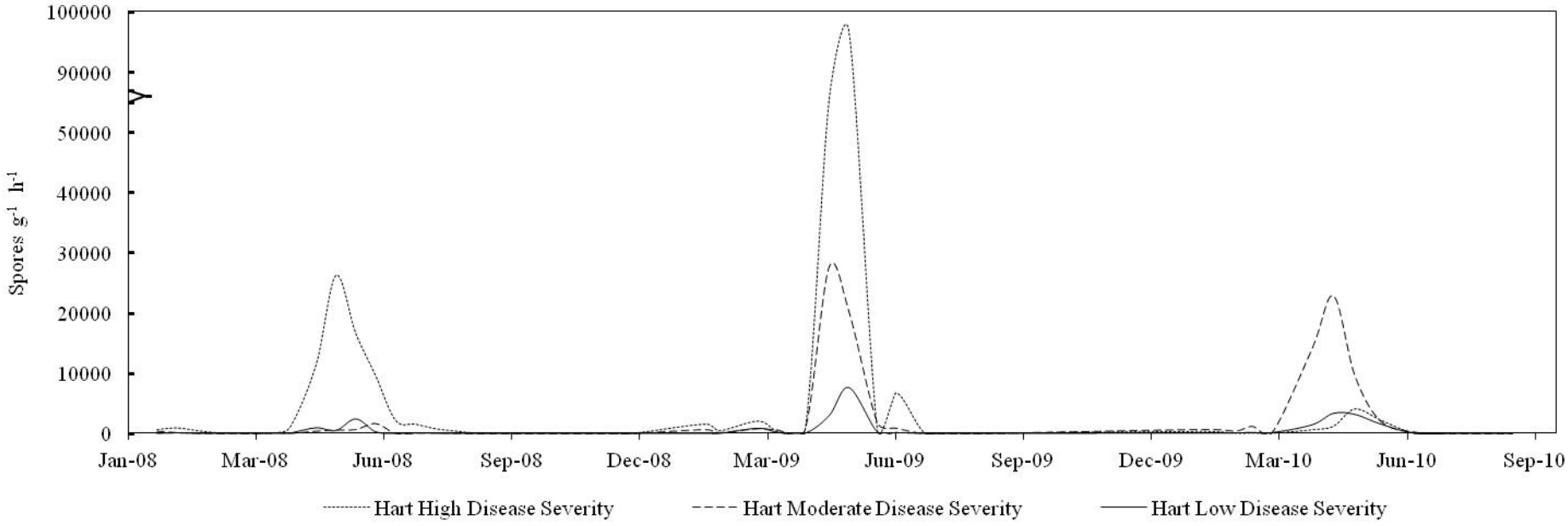

100000
90000
50000
40000
30000
20000
10000
0
Spores g-1 h-1
Jan-08
Mar-08
Jun-08
Sep-08
Dec-08
Mar-09
Jun-09
Sep-09
Dec-09
Mar-10
Jun-10
Sep-10
Hart High Disease Severity
Hart Moderate Disease Severity
Hart Low Disease Severity

Figure S3

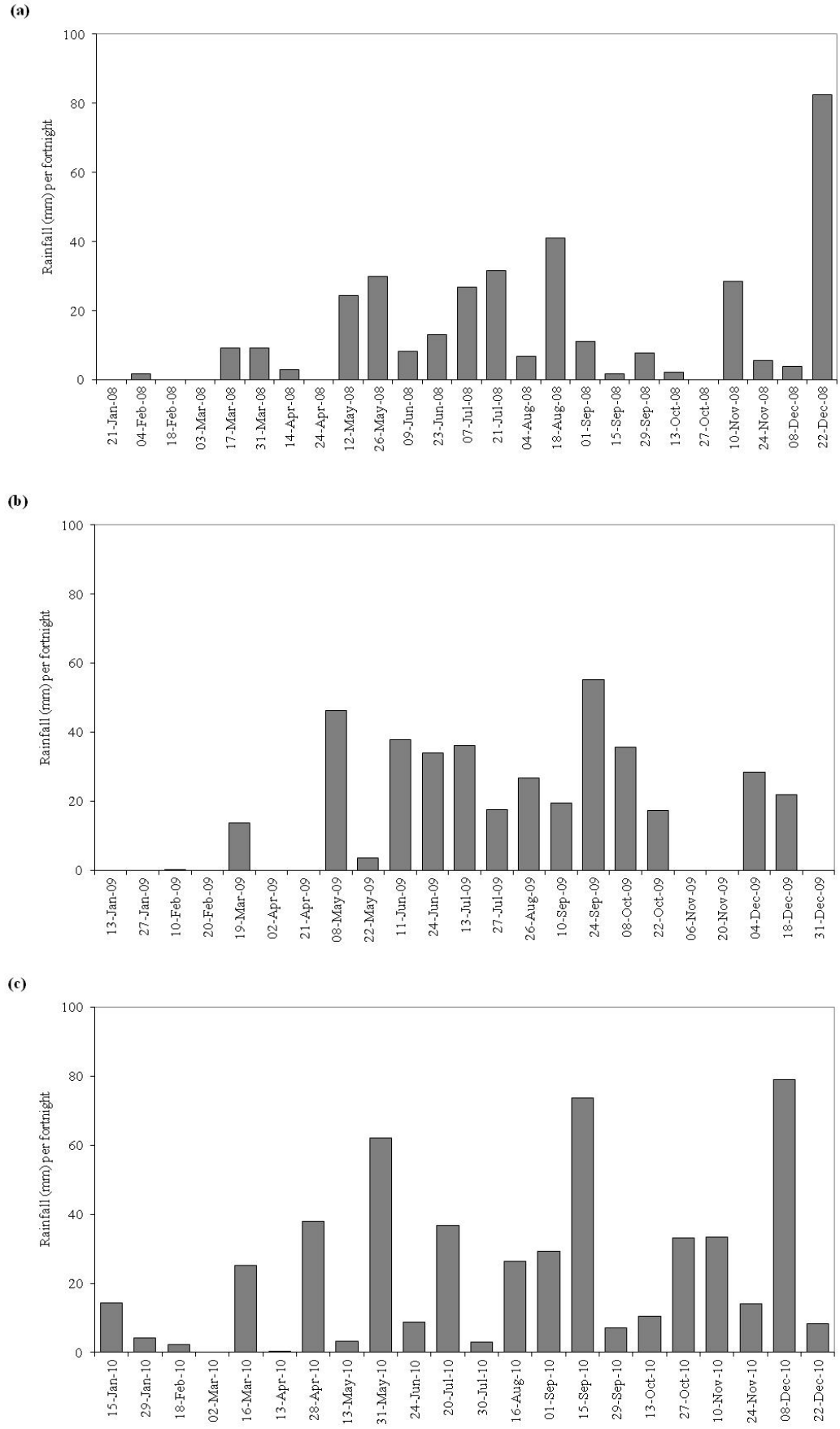

(a)
Rainfall (mm) per fortnight
100
80
60
40
20
0
21-Jan-08
04-Feb-08
18-Feb-08
03-Mar-08
17-Mar-08
31-Mar-08
14-Apr-08
24-Apr-08
12-May-08
26-May-08
09-Jun-08
23-Jun-08
07-Jul-08
21-Jul-08
04-Aug-08
18-Aug-08
01-Sep-08
15-Sep-08
29-Sep-08
13-Oct-08
27-Oct-08
10-Nov-08
24-Nov-08
08-Dec-08
22-Dec-08
(b)
Rainfall (mm) per fortnight
100
80
60
40
20
0
13-Jan-09
27-Jan-09
10-Feb-09
20-Feb-09
19-Mar-09
02-Apr-09
21-Apr-09
08-May-09
22-May-09
11-Jun-09
24-Jun-09
13-Jul-09
27-Jul-09
26-Aug-09
10-Sep-09
24-Sep-09
08-Oct-09
22-Oct-09
06-Nov-09
20-Nov-09
04-Dec-09
18-Dec-09
31-Dec-09
(c)
Rainfall (mm) per fortnight
100
80
60
40
20
0
15-Jan-10
29-Jan-10
18-Feb-10
02-Mar-10
16-Mar-10
13-Apr-10
28-Apr-10
13-May-10
31-May-10
24-Jun-10
20-Jul-10
30-Jul-10
16-Aug-10
01-Sep-10
15-Sep-10
29-Sep-10
13-Oct-10
27-Oct-10
10-Nov-10
24-Nov-10
08-Dec-10
22-Dec-10

Figure S4

Chapter 7.

General discussion

GENERAL DISCUSSION

This study has provided new information on the biology and control of ascochyta blight of field pea in southern Australia. Investigation of the new high yielding cultivars adapted to southern Australia provided new management options for disease control as they were less susceptible to yield loss when sowing was delayed than are existing cultivars. The fungus, *Phoma koolunga*, was described and recognised for the first time as an important component of the ascochyta blight complex on field pea in southern Australia. The distribution of *P. koolunga* in field pea cropping soils of this region and the longevity of the pathogen in the soils was compared to that of the other important causal pathogens of this disease complex, namely *D. pinodes* and *P. medicaginis* var. *pinodella,* and the competitiveness of these pathogens was compared in controlled conditions. Analysis of release of ascospores of *D. pinodes* at crop emergence identified a direct relationship with disease severity up to a threshold number, above which the severity of ascochyta blight did not increase.

The differentiation of *P. koolunga* from *D. pinodes, P. medicaginis* var. *pinodella* and *A. pisi*, and also from *M. phaseolina*, on the basis of ITS sequences and morphology (see Chapter 2) contributed to improved knowledge of the pathogens that cause ascochyta blight on field pea in South Australia and provided a basis for comparison of these pathogens. *P. koolunga* has not been identified in any other part of the world and its origin is unknown. Findings that *P. koolunga* was more common in South Australia than in Victoria, New South Wales or Western Australia and that its distribution in soil was closely linked to field pea cropping suggest that it may have originated in South Australia. Although the lower rate of detection in the other states indicates that *P. koolunga* may have been transported there on field pea seed, seed to seedling transfer of this pathogen has not yet been examined. Investigation of seed to seedling transmission and of the host range of *P. koolunga,* involving

native plant species of, as well as pasture and crop species grown in southern Australia, is required to understand how this pathogen has risen to prominence in this region.

Phylogenetic comparison of *P. koolunga* and the other causal agents of ascochyta blight, as well as other *Phoma* and *Ascochyta* species, may assist in determining the origin of this pathogen. For example, a single recent founder event for *A. rabiei* in Australia was inferred from the low genetic diversity identified among 104 isolates of this pathogen collected across the Australian chickpea growing area (Leo *et al.* 2012). In the current study (see Chapter 2) the ITS sequences of 13 isolates of *P. koolunga* were identical, and grouped into one clade separate from the other pathogens examined. The close evolutionary relationship between *D. pinodes* and *P. medicaginis* var. *pinodella* was also evident as they were grouped together in a single clade while *A. pisi* was placed in a separate clade with *A. fabae* and *A. lentis.* A similar study with a much larger collection of isolates of *P. koolunga* from diverse geographical locations and alternative hosts (if found) plus other *Phoma* species, and including analysis of sequences in addition to the ITS region, would assist in identifying its relationship to these other pathogens.

The DNA tests used here (see Chapters 2, 4 and 5) were based on the ITS region and, since *D. pinodes* and *P. medicaginis* var. *pinodella* are identical in that region, it was not possible to distinguish between these two pathogens. An assay that could distinguish between them would facilitate interpretation of DNA quantification in both plants and soil samples. Complete genomic sequencing may enable identification of sequences unique to each of these pathogens. Chilvers *et al.* (2009) have demonstrated that *P. koolunga* is phylogenetically distinct from, but related to, the known ascochyta blight pathogens of field pea, through analysis of partial sequences for RNA polymerase II subunit 2 (*RPB2*) and glyceraldehyde-3-phosphate dehydrogenase (*G3PD*) regions. In 2007, Peever *et al*. inferred evolutionary relationships among a worldwide sample of *Ascochyta* fungi based on analysis of DNA sequences, and identified that *A. pinodes* (teleomorph *Didymella pinodes*) clustered with

Didymella species and not with the original genus to which it was assigned *viz. Mycosphaerella*. These authors also found that isolates of *Ascochyta* species clustered into clades specific for the host of origin, *viz*. field pea, lentil, faba bean and chickpea, suggesting co-evolution of pathogen and crop. A similar study should be undertaken to determine if there is an evolutionary link between *P. koolunga* and its host(s).

Large collections of isolates are needed to understand the variability in pathogen populations. It is important to select appropriate isolates for resistance screening, given that particularly aggressive isolates can mask partial resistance to ascochyta blight in field pea (Onfroy *et al.* 1999). That *P. koolunga* caused less severe disease than *D. pinodes* may have been because the isolate used in that experiment (DAR78535) was the least aggressive of the 10 *P. koolunga* isolates tested (see Chapter 2). However there are also significant differences in aggressiveness among isolates of *D. pinodes* (Wroth 1998b) and the status of the isolate used was not known; this could also have influenced results. Consequently, further research on variability within *P. koolunga* is warranted, including comparison with a range of isolates of *D. pinodes* chosen to represent the diversity of aggressiveness within this species.

As the severity of disease caused by *P. koolunga* was influenced by a significant and consistent interaction between isolates and pea lines (see Chapter 2), further investigation is required to confirm that heritable resistance to *P. koolunga* exists in the field pea germplasm and whether it is the same or similar to the complex mechanisms that confer resistance to *D. pinodes* (summarised in Muehlbauer and Chen 2007). Developing resistant field pea cultivars will become even more challenging in southern Australia now that *P. koolunga* has been recognised as adding to the complexity of the pathogens involved. Little research has been conducted on resistance to *P. medicaginis* var. *pinodella* (Hillstrand and Auld 1982, Knappe and Hoppe 1995, Sakar *et al.* 1982), possibly because it often causes less disease than *D. pinodes* (Muehlbauer and Chen 2007, Onfroy *et al*. 1999, see Chapter 2). However, Knappe and Hoppe (1995) concluded it to be more aggressive than *D. pinodes* on pea epicotyls and,

unlike *D. pinodes*, single gene resistance has been identified (Rastogi and Saini 1984). It was shown here that *P. medicaginis* var. *pinodella* was often a minor component of the ascochyta blight complex in southern Australia making up only 5 % of the 697 isolates obtained over 3 years of sampling (see Chapter 4). However, it was detected at a frequency of 20 % of isolates at the medium-high rainfall site in 2007, suggesting that this pathogen might be responsible for severe disease in individual situations. Consequently, its role in the disease complex cannot be ignored, and research on *P. medicaginis* var. *pinodella* to understand the circumstances that lead to severe disease is warranted.

P. koolunga made up a substantial proportion of the pathogens present in the naturally infected field pea plants collected from the disease management trials, both in frequency of isolation and quantity of DNA, but was detected less often than *D. pinodes* plus *P. medicaginis* var. *pinodella* in soils across the field pea cropping regions of southern Australia. Additionally, *P. koolunga* was coincident with *D. pinodes* plus *P. medicaginis* var. *pinodella* in all but three soil samples from South Australia while the latter pair was regularly detected where *P. koolunga* was not (see Chapter 4). This may indicate that all three pathogens have a strong link to field pea cultivation, and that longevity of *D. pinodes* plus *P. medicaginis* var. *pinodella* was greater than *P. koolunga*. *D. pinodes* and *P. medicaginis* var. *pinodella* form chlamydospores as survival structures while *P. koolunga* produced pseudosclerotia in culture. The chlamydospores of the former pathogens are known to withstand temperatures of at least 100 °C for 12 – 15 h (Wallen *et al.* 1967b) and survive for over a year in soil (Wallen and Jeun 1968). Additional research is required to understand whether pseudosclerotia of *P. koolunga* are able to withstand the hot and dry conditions that occur in Australian summers between cropping seasons.

The frequency of isolation of all the pathogens was consistently low in the sandy loams of the low rainfall disease management trials at Minnipa in South Australia. Likewise, sandy soils in Western Australia are reported to contain very little soil-borne inoculum of

ascochyta blight pathogens (MacLeod *et al.* 2005) and investigating the effect of soil type on pathogen survival would assist in understanding the comparative longevity of the soil-borne inoculum following an ascochyta blight infested field pea crop.

The decrease in quantity of ascochyta blight pathogens over time (see Chapter 4) was in accordance with decrease in disease severity with increased intervals between field pea crops in rotations (Davidson and Ramsey 2000, McDonald and Peck 2009). The importance of crop rotation in managing ascochyta blight has been demonstrated elsewhere (Davidson and Ramsey 2000) and growers are encouraged to observe 5 years between field pea crops (Hawthorne *et al.* 2011). The PredictaB commercial service at SARDI (Ophel-Keller *et al.* 2008) now offers a soil test for *D. pinodes* plus *P. medicaginis* var. *pinodella* and for *P. koolunga* to assist growers to assess the soil-borne disease risk from ascochyta blight before planting a crop of field pea since these pathogens can exist in soil at high levels for up to 10 years (Davidson *et al.* 2001).

As was found for soil-borne inoculum, *D. pinodes, P. medicaginis* var. *pinodella* and *P. koolunga* were also co-located on field pea plants (see Chapter 4). It is common for plants to be simultaneously infected by more than one pathogen and, where the species occupy different niches, i.e. are separated in space, time or use of resources, they can co-exist indefinitely (Fitt *et al.* 2006). However, interactions occur when they occupy the same niche leading to antagonism where they have a negative effect on each other, or synergism where one pathogen promotes the growth of the other (Le May *et al.* 2009); both of these phenomena were observed among combinations involving *P. koolunga* (see Chapter 5). The results presented here also indicated that environment influences this interaction, demonstrated by the varying results when excised leaf discs were supported on water agar or in sterile water. Experiments in which plants are co-inoculated with *P. koolunga* and *D. pinodes* or *P. medicaginis* var. *pinodella* at different times and exposed to a range of

environmental conditions will assist in identifying the dominant coloniser or conditions in which one or the other may be dominant.

Although *P. koolunga* was recognised as an important component of the ascochyta blight complex on field pea, *D. pinodes* remains the principal pathogen of concern in South Australia and elsewhere, mainly due to the airborne ascospores that constitute the primary inoculum during crop establishment. Airborne spores have not been identified in association with the other pathogens in this disease complex, which instead appear to rely on rain splash of *in situ* inoculum for primary infection. Traditionally, growers were advised to delay sowing field pea crops 4 - 6 weeks beyond the first autumn rains to minimise exposure to the ascospores (Bretag *et al.* 2000, Davidson and Ramsey 2000, Hawthorne *et al.* 2011). This study established that there was a clear relationship between numbers of ascospores and disease severity up to a threshold number of spores, after which there was no further increase in disease (see Chapter 6). This threshold was small compared to the total number of ascospores released each season and small in comparison to the variation in number of spores released each season and from stubble samples with different amounts of disease. Hence avoiding the ascospores of *D. pinodes* where possible remains an important strategy to reduce the incidence and severity of ascochyta blight of field pea in Australia, and the temporal release pattern predicted by 'G1 Blackspot Manager' assists with this practice (Salam *et al.* 2011b). Delayed sowing to avoid the major window of spore release has also been suggested as a means of managing ascochyta blight of chickpea in the Pacific Northwest of the United States (Chilvers *et al.* 2007). As for *D. pinodes,* the pattern of spore release for *D. rabiei* was similar within each year for sites of close proximity, suggesting that local weather events influenced the release of primary inoculum and a model to identify optimum sowing dates was postulated for chickpea in that region.

The seasonal fluctuation in the total number of ascospores of *D. pinodes* detected was partially influenced by the disease severity on the infested field pea stubble; greater severity

resulted in more ascospores when the stubble samples were incubated in the same season at the same site. However, this relationship was not observed for stubble incubated in different seasons, and stubble with the greatest disease severity overall did not produce the highest number of ascospores (see Chapter 6). In this study, no account was taken of the spring release of ascospores within crops. It is possible that when large numbers of ascospores are released during spring, as secondary inoculum, the pathogen in the subsequent stubble produces relatively few ascospores as primary inoculum in the following autumn (See Chapter 6). Successive crops of mature pseudothecia are known to develop on field pea residues, initially copious, but the inoculum is exhausted within 50 weeks (Carter 1963, McDonald and Peck 2009, Peck *et al*. 2001). Exhaustion of inoculum has also observed in *Leptosphaeria maculans*, where 6-month-old canola stubble discharged 30-fold more ascospores per ha than older stubble (Marcroft *et al.* 2003). The effect of microclimate on ascospore release should also be taken into account. For example, in the cases of *D. rabiei* (Chilvers *et al.* 2007) and *L. maculans* (Marcroft *et al.* 2003), the variation in numbers of ascospores released from stubble of the same source but incubated at different sites was attributed to the effects of microclimate. More ascospores of *L. maculans* were released in a high rainfall environment than from stubble of the same age in a medium rainfall environment, which in turn discharged more than stubble from the low rainfall environment (Marcroft *et al.* 2003).

The importance of ascospores as secondary inoculum was also demonstrated. Control strategies should be designed to reduce and delay as long as possible the production of secondary inoculum, particularly ascospores, (Roger and Tivoli 1996). Secondary inoculum increased with rainfall during late winter and spring, and had an important role in determining disease severity at the end of the season (see Chapter 6). The amount of secondary inoculum was also inferred by disease severity in the crop, so that early sown field pea with severe disease produced most secondary inoculum, leading to a compound effect on disease severity.

If disease management strategies are applied during early crop growth it is unlikely the disease will be severe when the crop matures in spring. This can be achieved through a combination of practices, including manipulating sowing dates and distance from infested stubble to minimise exposure to ascospores of *D. pinodes* (Salam *et al.* 2011a, see Chapter 6), rotations of at least 5 years between field pea crops to avoid *in situ* inoculum (see Chapter 4), and strategic application of fungicides where economic (see Chapter 3). In a survey conducted by Peck and McDonald (2001), two thirds of growers in the medium - high rainfall region of South Australia claimed to use 5 year rotations and delayed sowing to reduce the severity of ascochyta blight in field pea crops. However in the current study, basic agronomic strategies of distance from stubble and rotations between crops were ignored by growers in the medium rainfall zone (see Chapter 6). Possibly, this is due to constraints for field selection based on suitable soil type and rotations on a single property, although field pea cropping intensity in both these regions is stable at approximately 5 % of area (Fulwood 2010, McMurray and Seymour 2005).

As reported following similar research in Canada (Wallen 1964, Warkentin *et al.* 1995, Warkentin *et al.* 2000), fungicide treatments only provided a small amount of protection from ascochyta blight and were uneconomic when hot and dry conditions, which minimised the spread of disease in spring, occurred during flowering and grain fill (see Chapter 3). However, yield increases in response to fungicide applications were observed when high rainfall favoured the development of ascochyta blight, demonstrating that in countries such as Canada and Australia the seasonal rainfall must be considered before applying fungicide (Xue *et al.* 2003). In Europe where yield and hence profit from field pea is higher, protectant foliar fungicides can be applied according to threat of disease (Roger and Tivoli 1996b, Roger *et al.* 1999a). The treatments in the current study were limited to the chemicals registered for field pea in Australia, *viz*. the foliar fungicides chlorothalonil and mancozeb and the seed dressing P–Pickel T®, and economic analysis determined that only

two foliar sprays were affordable in field pea crops. However, improved disease control and higher yields were achieved with fortnightly applications of chlorothalonil (see Chapter 3), illustrating that fungicides with a longer period of protection need to be identified and registered for the control of ascochyta blight in field pea. Many chemicals become less costly when their patents are no longer active so, in time, lower chemical costs and increased yield from advanced field pea germplasm should improve the economic gain from foliar fungicide. An increase in the cost-benefit ratio may also allow multiple applications of fungicide within the crop to control the primary and secondary inoculum spore showers similar to the strategic fungicide sprays that Australian growers regularly apply to more valuable pulse crops, namely, lentil, chickpea and faba bean (Davidson and Kimber 2007).

Crops grown in short growing seasons and or lower rainfall had a greater risk of yield loss from drought stress during crop maturation than from ascochyta blight (Armstrong *et al* 2008, Heenan 1994, Frischke and McMurray 2001). This is managed in consistently low rainfall regions, such as Minnipa, by sowing as early as possible, irrespective of the disease risk. In these areas, fungicides are not economic and, as such, field selection is vital to distance the crop from infested stubble. Nevertheless, when these regions experience more rain than normal during the growing season, ascochyta blight can be severe. This occurred at Minnipa in 2009 when 65 % of ascospores were still present at crop emergence and annual rainfall was 156.8 % above the average rainfall of the two preceding seasons (see Chapter 3), leading to severe disease (more than 12 nodes affected) in the earliest sown plots. Hence sowing in response to the predicted time of ascospore release is still important in this region but a different threshold level may need to be calculated. Recent improvements in long range (3-month) weather forecasting by the Australian Bureau of Meteorology (www.bom.gov.au/climate) may also assist in identifying seasons when delayed sowing in the lower rainfall regions might be beneficial. However, in medium rainfall and medium - high rainfall regions of southern Australia, the risk of yield loss from disease is often higher than

yield loss from drought stress; but the risk changes from season to season. This poses the difficulty of whether a grower should be advised to sow later to avoid ascochyta blight, risking yield loss in a dry spring, or to sow earlier and possibly lose yield through disease, again emphasising the need for improvement in long range weather forecasting. 'G1 Blackspot Manager' (Salam *et al.* 2011b) removes some of this risk by identifying those seasons in which the ascospores are released early and early sowing can proceed with low disease risk. The risk of low yield from later sowing has also been reduced through the release of new field pea cultivars from the Pulse Breeding Australia's Field Pea Program. The breeding line OZP0602, subsequently released as Gunyah, was shown in this research (see Chapter 3) to have an earlier and longer flowering period than the widely grown cultivars Kaspa and Parafield. This flowering period meant that Gunyah was less susceptible to yield loss when sowing was delayed, and yield was similar in the disease management field trials when it was sown either 3 or 6 weeks after the opening rains.

In conclusion, ascochyta blight must be managed in South Australian field pea crops as all cultivars are susceptible and the disease remains widespread. Strategic foliar fungicides were economic only when disease risk was high and crop yield was likely to be above 2 t ha^{-1} and, consequently, alternative chemicals and control strategies are required. This study has provided new information on the importance of soil-borne inoculum, and the demonstrated prevalence of *P. koolunga* in soils has led to the development of a commercial soil test for predicting disease risk prior to sowing. While there is much to be learned about the biology of the recently identified *P. koolunga,* this research suggest it competes with the other ascochyta blight pathogens for access to space and or nutrients on the plant. Identification of the relationship between ascospore load of *D. pinodes* and disease severity led to the recommendation that growers should sow field pea according to the temporal release of ascospores. As new cultivars arising from germplasm with earlier and longer flowering periods are released, the sowing date can be delayed with less risk of yield loss from hot dry

conditions during crop maturation. The newly released Gunyah showed a slight increase in ascochyta blight resistance compared with Kaspa but this was insufficient to prevent yield loss from disease. In the longer term, increased disease resistance in germplasm with long flowering periods should improve the flexibility to sow early or late depending on ascochyta blight risk to maximise yield of field pea in these regions where crops are threatened by ascochyta blight.

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(Literature Review and General discussion)

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