Aspects of epidemiology of *Phoma koolunga* (ascochyta blight of field pea)

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Abstract

Ascochyta blight (blackspot) is a significant disease of field pea (*Pisum sativum*) with worldwide distribution, causing grain production losses of 15 % per annum in Australia. *Phoma koolunga* is a relatively new pathogen of this complex disease in Australian field pea crops. This thesis reports information about aspects of the epidemiology of this fungus in Australian conditions.

The survival of *P. koolunga* on field pea stubble and as pseudosclerotia buried in field soil was examined. The frequency of recovery of this fungus declined over time and it was not recovered from stubble buried in soil or placed on the soil surface in pots outdoors at months 11 and 15, respectively, and later. Pseudosclerotia were produced in Petri dishes containing potato dextrose agar (PDA) amended with fluorocytocin or with sand. The maximum longevity of pseudosclerotia buried in soil in pots outdoors was less than 18 months. Infectivity of inoculum of the fungus decreased over time, as the mean number of lesions on plants inoculated with stubble buried or left on the soil surface for up to 6 and 5 months, respectively, and pseudosclerotia retrieved at 14 months and later from field soil did not differ from the water control in a pot bioassay.

P. koolunga was isolated from field pea seed samples harvested from South Australia and Victoria. Disease was transmitted to 98 % of seedlings that emerged from artificially inoculated seeds (AIS) in growth room conditions. Seedling emergence rate from AIS at 8° C soil temperature was lower than at 12, 16 and 20° C and also disease severity on seedlings was greater at the lower temperature. Efficacy of fungicides as seed dressings was examined on AIS. P-Pickel T® and Jockey Stayer® were the most effective fungicides among six tested for reducing

disease incidence and severity due to *P. koolunga* on seedlings that emerged from AIS sown in soil and on germination paper, respectively.

The reaction of 12 field pea genotypes to one moderately virulent isolate of *P. koolunga* was evaluated by spraying a pycnidiospore suspension on plants in controlled conditions and assessing disease severity at 2-5 day intervals for 21 days. Sturt, Morgan and Parafield showed more severe disease on leaves than the other genotypes at 21 days post-inoculation (dpi), and Kaspa, PBA Twilight, PBA Oura, PBA Wharton and WAPEA2211 were less susceptible. When three isolates of *P. koolunga* which varied in virulence were sprayed on four genotypes of short, semi-leafless type peas, Morgan and WAPEA2211 showed more disease than Kaspa at 21 dpi. Aggressiveness of isolates of *P. koolunga* on these four genotypes differed based on % leaf area diseased up to 14 dpi, but this difference had disappeared by 21 dpi.

Some isolates of *P. koolunga* from seeds showed atypical morphology and reproductive behaviour. These cultures had rhizoid form mycelia on growth media such as PDA. Also, some atypical cultures of *P. koolunga* sectored from typical colonies on PDA. These sectors and cultures were confirmed as *P. koolunga* by DNA test using *P. koolunga*-specific primers. Mycelium from these sectors produced small lesions on leaves and stems of field pea seedling resembling ascochyta blight symptoms in controlled conditions. Pycnidium-like structures of these atypical cultures did not contain pycnidiospores, but had many round and hyaline fatty guttulae of different sizes, usually smaller than normal spores of *P. koolunga* which never germinated.

Crossing 19 isolates of *P. koolunga* in vitro failed to initiate formation of pseudothecia of *P. koolunga* on pea stem pieces or on several growth media. This

fungus might need specific environmental conditions for production of pseudothecia which still are unknown.

The results of this study provide information about survival of *P. koolunga*, transmission to seedlings via infected seed, control of the fungus in seed and also reaction of field pea genotypes to this pathogen in South Australian conditions. These findings improve the understanding of epidemiology of this disease and consequently can help to improve management of this pathogen in the field.

Declaration

I, Mohsen Khani, certify that 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. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Mohsen Khani……..

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Chapter 1

Introduction and literature review

1.1 Introduction

Ascochyta blight (synonym: blackspot) is a devastating foliar disease of field pea (*Pisum sativum* L.) in Australia (Ali & Dennis, 1992) and around the world, particularly in Europe, New Zealand and North America. It has also been reported from Africa, and South and Central America (Bretag & Ramsey, 2001). Ascochyta blight is considered the most important disease of field pea in Australia, and Bretag (1991) estimated that the annual yield loss due to this disease was 15% across the entire Australian field pea production. However, individual crops can suffer up to 75% yield loss in severe cases (Bretag et al., 1995a, Davidson & Ramsey, 2000, Bretag & Ramsey, 2001, Salam et al., 2011c, Davidson, 2012). This disease is usually referred to as the ascochyta blight complex, because it is caused by a number of closely related fungal species which exist independently of each other. Until 2009, three species were known as causal agents of ascochyta blight, i.e., *Didymella pinodes* (syn. *Mycosphaerella pinodes*) as the predominant causal agent of this complex, *Phoma medicaginis* (Marlbr. & Roum.) var. *pinodella* (Jones) and *Ascochyta pisi* Lib. Davidson et al. (2009a) characterised *Phoma koolunga* as a fourth fungal species which can cause ascochyta blight on field pea. *Phoma herbarum* has also been reported to cause ascochyta blight-like symptoms on field pea at one site in Western Australia (Li et al., 2011). Although *Macrophomina phaseolina* had been reported to cause leaf, stem and petiole spots characteristic of ascochyta blight on pea field (Ali & Dennis, 1992), Davidson et al. (2009a) suggested that those isolates were likely to have been *P. koolunga* rather than *M. phaseolina*.

All parts of field pea plants can be infected by these pathogens, which results in necrotic spots on leaves, stems, pods, crowns and roots (Bretag et al.,

2006) and in cases of severe infection, seedling death can result (Xue et al., 1997). The pathogens can survive on field pea stubble in the field, on seeds and in soil (Skolko et al., 1954, Wallen & Jeun, 1968, Zhang et al., 2005).

Many foliar fungicides have been developed to control ascochyta blight on field pea, but they are usually costly to the farmer and often uneconomic (Warkentin et al., 1996, 2000, McMurray et al., 2011). Although, the development of ascochyta blight resistant pea genotypes is suggested as being the best long term strategy for disease control, so far, no completely resistant genotype has been developed (Bretag et al., 2006). Therefore, disease control relies on cultural methods. Practical control of ascochyta blight can be achieved by reducing primary inoculum, such as infested field pea stubble, infected seed and soil-borne inoculum (Bretag et al., 2006). Consequently, burying infested field pea stubble, rotation after field pea crops and delay in sowing seed to avoid the peak release of spores from stubble are considered the most important practical methods to manage this disease (Peck et al., 2001, Bretag et al., 2006, McDonald & Peck, 2009, Yeatman et al., 2009, Davidson, 2012).

Although much research has been published on *Didymella pinodes, Phoma medicaginis* var. *pinodella* and *Ascochyta pisi,* due to its recent discovery, many aspects of the biology and epidemiology of *P. koolunga* remain unknown.

1.2 Field pea

Field pea is one of the most important cool season food legume crops worldwide. It has a protein concentration of about 23%, making it a valuable source of protein for human food and livestock feed (Hawthorne, 2006). Field pea, by its ability to form a symbiotic relationship with *Rhizobium* bacteria, can fix atmospheric

nitrogen in root nodules, and the nitrogen remaining in the soil after harvest increases the yield of crops that are cultivated after field pea (Evans et al., 2001). For example, Evans et al. (2001) showed that if field pea was grown in rotation with wheat, then nitrogen mineralized from field pea residues contributed 15-30% of the nitrogen to the average grain yield of the following wheat crop. Hence by cultivating field pea in rotation with other crops, the need to use synthetic nitrogen fertilizer can be decreased by up to 30%, thereby reducing the cost of production of the subsequent cereal crop.

The area harvested, yield and production of field pea in 2012 were estimated to be 6.3 million ha, 1.55 tonne/ha, 9.9 million tonnes in the world, and 282,700 ha, 1.36 tonne/ha and 385,600 tonnes in Australia (Food and Agriculture Organization of the United Nations, 2011). South Australia grows approximately half of the field pea in Australia with 113,500 ha harvested, average yield of 1.51 tonnes/ha and 172,000 tonnes produced in 2012 (Pulse Australia, 2012).

The main field pea genotypes planted in southern and Western Australia are the semi-leafless types, such as the genotype Kaspa. This is a broadly adapted semi-dwarf field pea with lodging and shatter resistance, grown in areas with medium to high rainfall (400-500 mm per year) while cv. Parafield, a tall conventional pea genotype, is more suited in regions with lower rainfall (Kennedy et al., 2013, Pulse Australia, 2013).

1.3 Ascochyta blight pathogens

As noted above, several fungal species can be responsible for ascochyta blight of field pea*; viz. Didymella pinodes*, the predominant species in this complex, *Phoma medicaginis* var. *pinodella*, *Ascochyta pisi* (Bretag & Ramsey, 2001), the

newly characterised *Phoma koolunga* (Davidson et al., 2009a) and, in one situation, *P. herbarum* (Li et al., 2011).

Aveskamp et al. (2010) have suggested that *Didymella pinodes* and *Phoma medicaginis* var. *pinodella* be re-named *Peyronellaea pinodes* and *Peyronellaea pinodella*, respectively, based on DNA analyses but the names *D. pinodes* and *P. medicaginis* var. *pinodella* will be retained in this thesis as most references use these terms.

Davidson et al. (2011), in a survey on distribution and survival of ascochyta blight pathogens in Australia, found that *P. koolunga* was most common in South Australian field pea growing areas but detected it in comparatively few soil samples from Western Australia, Victoria and New South Wales. They suggested that this may show that *P. koolunga* originated in South Australia and has recently been distributed to other states. They also predicted that this species may become more widespread in the future.

All species can be found together on the host or occur as single pathogens independently (Tivoli et al., 2006, Davidson et al., 2009a). Davidson et al. (2011) showed that at three locations in South Australia in 2007 and 2008 with varying annual rainfall, *D. pinodes* constituted 54% of the 697 isolates cultured from ascochyta blight lesions while 41% were *P. koolunga* and 5% *P. medicaginis* var. *pinodella*; *A. pisi* was not isolated. In comparison, Ali and Dennis (1992) stated that *P. medicaginis* var. *pinodella* and *D. pinodes* were common in South Australia, whereas they rarely found *A. pisi*.

Colonies of *D. pinodes* on culture media such as malt agar (MA), oatmeal agar medium (OA) or V8 medium, in general, are light to dark grey, with pycnidia

and pseudothecia usually formed along the radii of mycelium around the plug. Under a 12 h photoperiod, when colonies reach 20-30 mm in diameter, the pycnidia and pseudothecia grow in concentric zones (Onfroy et al., 1999). Pycnidia formed on stems are 100-200 μm in diameter with hyaline conidia which have one or rarely two septa, and are 8-16 × 3-4.5 μm (Punithalingam & Holliday, 1972b). In comparison, colonies of *P. medicaginis* var. *pinodella* on MA are darker grey at the beginning of growth, then turning to black at maturity, mostly due to production of numerous chlamydospores. In response to 12 h photoperiod, mycelium and pycnidia grow in concentric rings around the initial mycelial plug in plates (Onfroy et al., 1999). Pycnidia are sub-globose to variable in shape, 200-300 μm in diameter. Conidia are smaller than *D. pinodes* conidia at 4.5-8 (-10) × 2-3 μm, hyaline, usually unicellular, occasionally with one septum. Chlamydospores of *P. medicaginis* var. *pinodella* are dark brown, spherical to irregular, smooth to rough, terminal or intercalary and produced singly or in chains (Punithalingam & Gibson, 1976). Pycnidia of *A. pisi* on leaves and pods are globose and brown, 100–200 μm in diameter. Conidia are hyaline, straight or slightly curved, one-septate, slightly constricted at the septum, shortly cylindrical with rounded ends, 10–16 × 3–45 μm. Colonies on OA produce abundant pycnidia with carrot red exudate (Punithalingam & Holliday, 1972a). Colonies of *P. koolunga* on MA after 7 days are approximately 12 mm in diameter, irregular with compact cottony white to pale grey aerial mycelium, occasionally dark olivaceous with little or no aerial mycelium, reverse yellow-brown to olivaceous. Pycnidia are globose usually with an elongated neck, solitary, infrequently confluent, scattering on the agar or immersed, 150-210 μm in diameter. Conidia are hyaline, ellipsoid to oblong, 12.5-17 × 5-7 μm, mainly aseptate, occasionally

with one septum. This fungus does not produce chlamydospores, but pseudosclerotia may be present, usually on radiating lines of mycelium (Davidson et al., 2009a). Therefore, *P. koolunga* is distinguishable from the other ascochyta blight causal agents by its comparatively large unicellular conidia and cultural growth (Davidson et al., 2009b).

Among the pathogens causing ascochyta blight, the teleomorph of the three previously described species have been reported. *Didymella pinodes* is the teleomorph of *Ascochyta pinodes*, and forms pseudothecia on stems and pods of field pea. These pseudothecia are globose with 90×180 μm diameter, dark brown, and contain cylindrical to subclavate and bitunicate asci. Ascospores are hyaline with ends rounded and one-septate, 12-14×4-8 μm (Punithalingam & Holliday, 1972a, Peever et al., 2007).

The teleomorph of *Phoma medicaginis* var. *pinodella* was reported on infected plant material in controlled conditions (Bowen et al., 1997). This fungus produced globose, dark brown pseudothecia, 140-250×170-420 μm in diameter with papillate ostioles and contained cylindrical to subclavate and bitunicate asci 140-290×20-30 μm. Eight hyaline, ellipsoid, bicellular ascospores, rounded at the end and 12.5-19×25-35 μm were formed in each ascus. However, this teleomorph has never been detected in natural conditions and other researchers have been unable to reproduce the teleomorph (Onfroy et al., 1999, Tivoli and Banniza, 2007).

Chilvers et al. (2009) described the sexual fruiting bodies of *A. pisi* formed in laboratory conditions and named the teleomorph *Didymella pisi*. They reported that after 8 weeks at 10°C, mature pseudothecia were rounded to irregular, brown to black with inconspicuous ostiole, and about 200-400 μm in diameter. Those

pseudothecia produced cylindrical to sacate and bitunicate asci, 10-15×46-168 μm. Eight hyaline and usually equally bicellular ascospores were formed in each ascus. The ascospores were rounded at both ends but one end was more acute and their size was about 17.5×6.5-8.5 μm. Neither pseudothecia nor ascospores have been reported for *A. pisi* or *P. medicaginis* on pea plants in the field (Bowen et al., 1997, Chilvers et al., 2009).

D. pinodes is homothallic (Punithalingam & Holliday, 1972b), unlike *A. pisi* which is heterothallic (Chilvers et al., 2009). Bowen et al. (1997) could not produce pseudothecia from pycnidiospores harvested from germination of single ascospores, therefore they suggested that *P. medicaginis* var. *pinodella* is heterothallic. So far, the teleomorph of *P. koolunga* has not been observed on growth culture media or on plant material. Also, no research has been done on *P. koolunga* to identify whether it may be homathallic or heterothallic.

1.4 Epidemiology of ascochyta blight

1.4.1 Symptoms and disease cycle

Initially *D. pinodes* forms small necrotic lesions on all aerial parts of field pea plants including leaf, stem, pod and seed; these expand and coalesce, and often cover entire leaves and pods. Stem lesions are produced at the base of dead leaves on plants and can girdle the lower parts of stems. Foot rot, the infection of the stem base, is another symptom caused by this pathogen. The symptoms caused by *P. medicaginis* var. *pinodella* are similar to those of *D. pinodes*. Deep necrotic lesions on the field pea stems can cause breaking of stems and lead to death of plant parts above the girdled part of the stem. These ascochyta blight pathogens are able to produce necrotic spots on pods that result in seed infection and, finally

declining quality of seed (Bretag & Ramsey, 2001, Tivoli et al., 2006, Hawthorne et al., 2012).

Davidson et al. (2009a) described symptoms caused by *P. koolunga* on field pea seedlings in a controlled environment as chlorotic and necrotic lesions 1-3 mm in diameter by 48 h after inoculation. These expanded and coalesced to cover entire leaves. Although the expansion rate of necrotic lesions caused by *P. koolunga* was similar to that of lesions formed by *D. pinodes*, the area of the lesions due to *D. pinodes* remained slightly larger for the duration of experiment. Lesions caused by *P. medicaginis* var. *pinodella* developed more slowly and they were observed on inoculated seedlings after 72 h, remaining roughly 1 mm in diameter. Davidson et al. (2009a) found the symptoms caused by *P. koolunga* to be indistinguishable from those caused by *D. pinodes*, except for a 24 h delay in development. The severity of disease caused by these two pathogens was similar at 7 days post-inoculation. All 10 isolates of *P. koolunga* tested by spraying of pycnidiospore suspension on four genotypes or breeding lines of field pea plants, *viz*. Kaspa, Alma, Parafield and WAPEA2211, caused extensive necrotic spots on leaves and internodes (Davidson et al., 2009a).

The disease cycle of ascochyta blight on field pea caused by *Didymella pinodes, Phoma medicaginis* var. *pinodella* and *Ascochyta pisi* is described in Fig. 1.1.

Ascospores of *D. pinodes* produced on infested plant debris and pycnidiospores of all pathogens of this complex produced on stubble or seeds, as well as soil-borne inoculum, are primary inoculum that can initiate disease on field pea plants. Ascospores spread over long distances via wind and rain, but can also be an important source of secondary inoculum when pseudothecia are formed on early

senesced leaves and spread within the crop, resulting in greater disease severity (Bretag, 1991, Tivoli & Banniza, 2007, Davidson et al., 2013). Pycnidiospores produced on plant surfaces during crop growth are dispersed as secondary inoculum over short distances and spread a maximum of 30 cm above the soil surface (Roger & Tivoli, 1996).

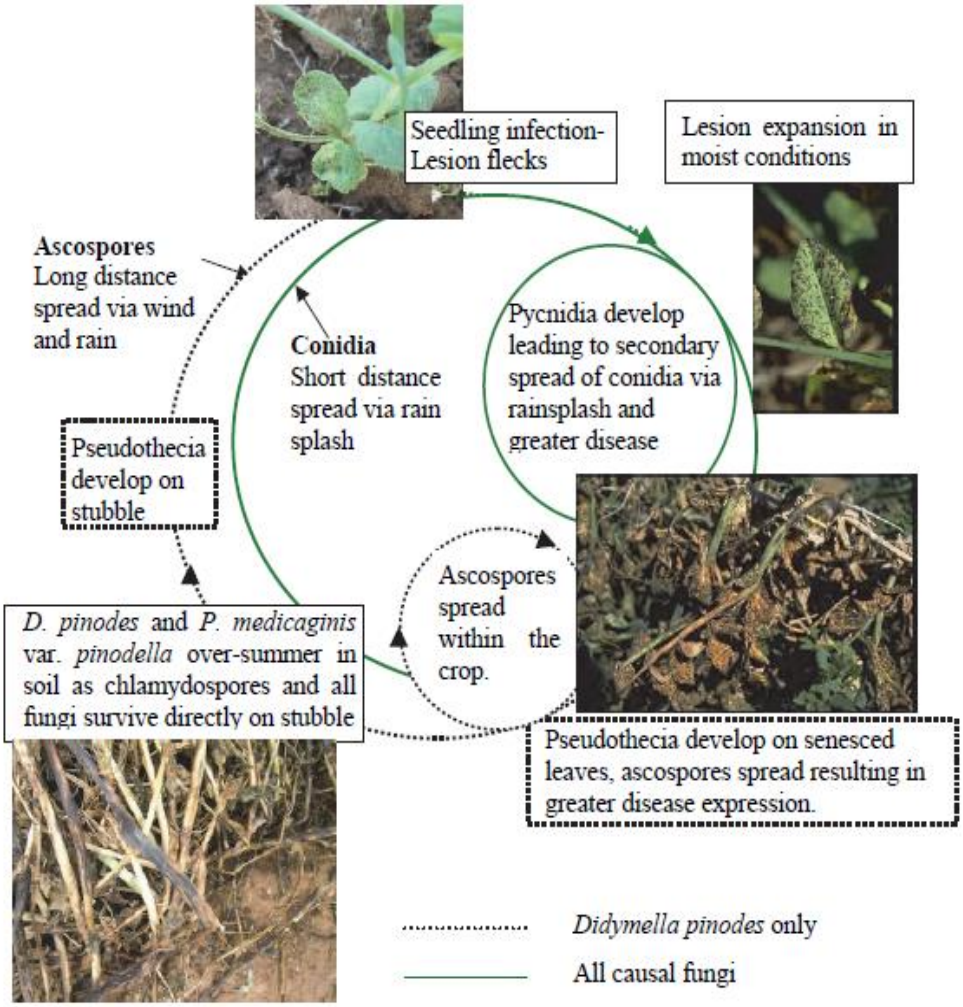

Figure 1.1 Disease cycle of ascochyta blight on field pea caused by *D. pinodes*, *Phoma medicaginis var pinodella* and *A. pisi* (Davidson, 2012)

The sexual state of a fungus not only produces spores as primary inoculum to initiate disease, but perhaps more importantly, it provides new combinations of

genes and hence new pathotypes that can be more virulent on particular hosts or resistant to commonly used fungicides (Bowen et al., 1997). When the teleomorph of a pathogenic fungus is present, disease control may need to be modified according to the time and amount of ascospores released in a season. Therefore, the possible formation of a sexual state of *P. koolunga* needs to be investigated.

1.4.2 Survival of ascochyta blight fungi

The major pathogen of the ascochyta blight complex, *D. pinodes*, is able to survive as pseudothecia or sclerotia on field pea stubble or as chlamydospores in infested soil for at least 12 months. In addition, the pathogen can survive on other hosts, if field peas are not grown, which provides primary inoculum to initiate disease in the next field pea crop (Bretag et al., 2006).

There is evidence that both incidence and severity of ascochyta blight increase if field pea crops are sown in the vicinity of infested field pea stubble (Davidson & Ramsey, 2000, Bretag & Ramsey, 2001, Galloway & McLeod, 2001, Bretag et al., 2006, Davidson et al., 2013). This is likely to be due to survival of the causal agents and production of pseudothecia and pycnidia on infested stubble (Dickinson & Sheridan, 1968, Sheridan, 1973, Davidson et al., 1999, Bretag & Ramsey, 2001, Peck et al., 2001, Zhang et al., 2005, Bretag et al., 2006, McDonald & Peck, 2009, Davidson et al., 2013).

In areas where ascochyta blight has occurred previously, infested field pea stubble is the main source of primary inoculum. In these areas, air-borne and rain-borne spores from infested residue or contact with soil-borne inoculum results in infection of new field pea crops (Bretag & Ramsey, 2001, Bretag et al., 2001, Galloway & McLeod, 2001, Bretag et al., 2006, Davidson et al., 2013). *D.*

pinodes has been reported to survive up to 4 months to 18 months on plant stubble. For example, in one report it was claimed that the pathogen survived less than 4 months in the absence of a host in New Zealand and it was concluded that the pathogen needs host residue to extend its survival (Cruickshank, 1952 in Zhang et al., 2005). Moreover, Davidson et al. (1999) reported that after 4 months, field pea stubble was not infectious and, therefore, was unlikely to lead to significant disease on field pea seedlings in South Australia. Zhang et al. (2005) showed that residue is very important for survival of *D. pinodes* and *P. medicaginis* var. *pinodella*, while it is not important for *A. pisi*, because of its poor saprophytic growth and competitiveness with other soil microorganisms (Dickinson & Sheridan, 1968). *D. pinodes* survived in non-buried stubble for more than one year in South Australia, depending on environmental conditions (Peck et al., 2001). McDonald and Peck (2009) showed that the amount of *D. pinodes* inoculum on pea stubble was high the year after a pea crop and declined rapidly after one season. In an experiment in Manitoba, Canada, Zhang et al. (2005) showed that the amount of spores (ascospores and pycnidiospores) produced on ascochyta blight infested field pea stubble declined over one year. In two different locations, Morden and Winnipeg, after 11 and 9 months, respectively, very few spores were produced from stubble which was buried at 5 and 10 cm below the soil surface or left on the soil surface. Additionally, Zhang et al. (2005) observed that spore production on residue on the soil surface was greater than that buried 5 and 10 cm below the soil surface. Consequently, disease severity on field pea seedlings inoculated with spores released from the residue on the soil surface was greater than those inoculated with spores from buried stubble.

Galloway and McLeod (2001) placed sets of field pea seedlings in pots at various distances from stubble infested by *D. pinodes* for intervals of one week and then, after incubating the pots in a glasshouse, measured disease severity on the seedlings. Their results suggested that sowing field pea at least 500 m distant from the previous year's stubble is the best means for reducing disease initiation from ascospores released from pseudothecia on stubble. Peck et al. (2001) also showed that, in South Australian conditions, infection of field pea seedlings from ascospores of *D. pinodes* released from field pea stubble was high, at 123 and 65 lesions on seedlings placed adjacent to and 400 m from the previous year's stubble, respectively, after 70-80 mm of rainfall recorded from 1st April. They observed that infection decreased to a very low level (close to 0 lesion) after the stubble was 12 months old. In another study in South Australia, Davidson et al. (2013) reported that ascochyta blight severity due to *D. pinodes* on field pea plants at crop maturity was related to the number of ascospores released from field pea stubble as well as secondary ascospore production within the crop. Also, they showed a linear relationship between the number of released ascospores from 0 to 294 and disease severity at the end of winter. Therefore, they identified 294 effective ascospores (per g stubble per ha) as a threshold amount of ascospores above which disease did not increase.

D. pinodes and *P. medicaginis* var. *pinodella* can survive in soil for at least 12 months by means of dormant mycelia, chlamydospores and sclerotia (Dickinson & Sheridan, 1968). Sheridan (1973) stated that mycelium of *D. pinodes* can grow saprophytically on field pea roots in the soil for nearly 18 months in competition with the indigenous soil microbiota in Ireland. Pycnidia or pseudothecia of *D. pinodes* in infested field pea stubble that is buried are

transformed into chlamydospores and it has been reported that more chlamydospores were produced after burial of stubble. Chlamydospores allow the fungus to survive in soil (Carter, 1961 in Bretag et al., 2006, Dickinson and Sheridan, 1968). Davidson et al. (2011) showed that, in a bioassay, the severity of ascochyta blight symptoms, including stem lesions and leaf spots, was positively correlated with the quantity of soil-borne inoculum of these pathogens measured by DNA tests.

As mentioned in section 1.4.1, the amount of rainfall is a crucial factor influencing disease severity on field pea plants and where the amount of soil borne inoculum is high enough, rain-splash transfers inoculum to the seedlings. A small amount of inoculum in the soil is thought to plays a minor role in disease severity compared to air-borne ascospores (Davidson et al., 2013).

Davidson et al. (2011) found a positive correlation between isolation frequencies of *Phoma koolunga*, *Didymella pinodes* and *P. medicaginis* var. *pinodella* from naturally infected field pea plants in disease management trials and the amount of fungal DNA detected in soil samples from the trial sites. These researchers stated that they could detect DNA of *P. koolunga* in field soil 4 years after a field pea crop was grown and predicted that the level of soil-borne inoculum was sufficient to initiate an ascochyta blight epidemic, but they did not indicate the means by which structures of this fungus might survive.

It has been claimed that *P. medicaginis* var. *pinodella* was isolated from field soil more than 17 years after the last rotation of field pea and this persistence was attributed to the presence of chlamydospores (Wallen, 1974). Peck et al. (2001) reported that infection of field pea from infested soil decreased slowly over 3 years. They concluded that soil-borne *D. pinodes* and *P. medicaginis* var.

pinodella could be considered as effective sources of inoculum for longer periods of time than fungi in stubble.

Although Davidson et al. (2009a) reported that *P. koolunga* may produce pseudosclerotia on yellow-brown to black-brown mycelium in culture media, pseudosclerotia have not been described in any detail. Also, the role of pseudosclerotia in life cycle of *P. koolunga* is not known. Pseudosclerotia, as the survival structures are reported for *Phoma ligulicola*, the causal agent of ray blight of pyrethrum (Blakeman & Hornby, 1966). Pethybridge et al. (2005) speculated that *P. ligulicola* was probably introduced into Tasmanian pyrethrum crops via pseudosclerotia in soil, epiphytic mycelium or via infested seeds. Those pseudosclerotia could survive in natural soil for up to 30 weeks whereas in compost they survived less than 8 weeks (Pethybridge et al., 2006). However, because rotation of at least 4 years between pyrethrum crops in the field is used in Tasmania, the importance of pseudosclerotia in survival between these crops was considered relatively minor (Pethybridge et al., 2006). Soil sterilisation can minimise the inoculum produced from pseudosclerotia of *P. ligulicola* (Fox, 1998). The relative significance of pseudosclerotia as secondary inoculum of *P. ligulicola* remains unknown (Pethybridge et al., 2008).

Alternative hosts can act as reservoirs of pathogen inoculum which can then be transferred to crop plants (Crous & Groenewald, 2005). Trapero-Casas and Kaiser (2009) isolated *Ascochyta rabiei* from three species of the Fabaceae and from 12 species of weeds in other families often found in commercial crops of chickpea. Bretag et al. (2006) stated that both *D. pinodes* and *P. medicaginis* var. *pinodella* are less specialised than *A. pisi* and invade a wide range of hosts, including species of *Pisum*, *Vicia*, *Lathyrus*, *Medicago*, *Vigna*, *Melilotus*, *Lupinus*,

Cicer, *Lens*, *Phaseolus* and *Trifolium*, while *A. pisi* has a smaller range of hosts in the genera *Pisum*, *Vicia*, *Lathyrus*, *Medicago*, *Cicer*, *Lens*, *Lupinus* and *Phaseolus*. In glasshouse conditions, *P. koolunga* can produce small lesions on some genotypes of *Lens culinaris*, *Medicago scutella* and and *M. littoralis* (Davidson et al., 2009b), but it is not known whether or not *P. koolunga* infects any of those above mentioned species in field conditions in South Australia. If so, how does this influence survival and distribution of *P. koolunga*?

Although there is much research published on the survival of ascochyta blight pathogens on field pea residues, there is little or no information about this subject for *P. koolunga*. Also, the role of chlamydospores and sclerotia of *D. pinodes*, and chlamydospores of *P. medicaginis* var. *pinodella*, in survival of these pathogens is well understood, but the importance of pseudosclerotia in the survival of *P. koolunga* is completely unknown.

1.4.3 Transmission of ascochyta blight pathogens via infected seeds

D. pinodes, *P. medicaginis* var. *pinodella* and *A. pisi* are seed-borne (Bretag et al., 1995b) and the use of infected seed leads to poor germination and death of seedlings due to foot rot (Wallen et al., 1967, Xue, 2000). Planting of field pea seed infected with *D. pinodes* often leads to symptoms developing on seedlings at or below the soil surface, especially at low temperatures (Moussart et al., 1998). However, seed infection by these pathogens rarely results in ascochyta blight epidemics (Moussart et al., 1998, Tivoli & Banniza, 2007). Nevertheless, the movement of infected field pea seed may introduce the disease or virulent pathotypes to new regions or countries (Moore, 1946, Tivoli & Banniza, 2007). Although seed to seedling transmission rate usually defines how many infected seeds develop into infected seedlings and this can affect an initiation of an

epidemic, Tivoli and Banniza (2007) concluded that the lack of correlation between seed infestation level and severity of disease caused by *D. pinodes* in research conducted by Bretag et al. (1995b) could be related to wind-borne ascospores masking the effect of seed infection. Bretag (1991) noted that varying levels of tolerance of infected seed were mentioned by different researchers to avoid ascochyta blight, ranging from 0 to 5%. Neergaard (1979) stated that in Canada seed lots with a maximum of 6% of seed infected by *A. pisi* and a total of 2% for *D. pinodes* and *P. medicaginis* var. *pinodella* together were allowed. In comparison, in a practical disease management bulletin for South and Western Australia (Hawthorne et al., 2012), seeds with less than 5% ascochyta blight infection were recommended as acceptable for sowing where clean seed was not available.

Three factors that can affect development of seed-borne diseases such as ascochyta blight are; (i) the quantity of seed-borne inoculum; (ii) seed to seedling transmission rate; and (iii) the rate of disease development in the field (Neergaard, 1979). Maude (1966) reported that up to 84% of seed infected by *D. pinodes* transferred disease to the seedling in favourable conditions (Bretag, 1991), while Xue (2000) observed that in controlled conditions seed to seedling transmission rate of this pathogen varied from 67-70% for healthy looking pea seeds which were harvested from naturally infected plants to 94-100% for visibly poor quality seeds with spots on them. Furthermore, he observed that emergence rate was 89-93% for healthy looking seeds and 19-23% for heavily infected seeds.

Ascochyta blight is more severe on field pea plants grown in wet weather, and seed produced in such conditions can be heavily infected, especially if rain occurs between flowering and maturity (Bretag, 1991). Also, the rate of seed to

plant transmission of ascochyta blight is much higher in wet conditions than in arid regions (Bretag, 1991). In addition to rainfall, late harvesting and early sowing of seeds can lead to frequent infection of seeds (Bretag et al., 1995b). Therefore, it is recommended that seed for sowing be accessed from areas with dry conditions or harvested as early as possible to avoid disease development in pods and seed (Hawthorne et al., 2012).

D. pinodes has been isolated from the embryo of grain from severely diseased field pea plants and pycnidia were observed in cotyledons inside seed (Moussart et al., 1998). Bretag et al. (1995b) showed that of 691 seed-lots tested from Victoria, Australia, 436 samples were infected by ascochyta blight pathogens, comprising 94.8% *D. pinodes*, 4.2% *Phoma medicaginis* var. *pinodella* and 1% *A. pisi*. Bretag et al. (2006), however, stated that some studies show storing field pea seeds for 7 years eliminated these pathogens, but this is not practical, mainly because it reduces the viability of seeds. Ali et al. (1982) demonstrated that 72% of seed samples of nine field pea genotypes from all regions of South Australia were infected by *Macrophomina phaseolina*, with 1-35% of seeds infected. Some or all of those isolates may have been mis-identified and, perhaps, were actually *P. koolunga* (Davidson et al., 2009a), so it is possible that *P. koolunga* is seed-borne. Furthermore, *D. pinodes* and *P. koolunga* can be equally responsible for ascochyta blight symptoms in South Australia (Davidson et al., 2011) and, unlike *D. pinodes*, *P. koolunga* does not produce ascospores. Hence, it is possible that seed infected with *P. koolunga* may act as a source of primary inoculum to initiate and spread disease in field pea crops in South Australia and elsewhere. Therefore, aspects of seed infection in relation to *P. koolunga* should be investigated to address this gap in knowledge.

1.4.4 Effect of ascochyta blight disease on field pea yield

Photosynthetic capacity of diseased leaves of field pea affected by ascochyta blight usually decreases and this reduction can decrease plant biomass or grain yield and quality (Beasse et al., 1999). Wallen (1965) artificially inoculated an equal amount of spores and mycelia of *D. pinodes*, *P. medicaginis* var. *pinodella* and *A. pisi* separately on field pea plants and reported that grain yield reduction due these pathogens was 45%, 25% and 11%, respectively.

It has been reported that yield reduction is linearly correlated to the final disease severity of ascochyta blight disease, to Area Under the Disease Progress Curve (AUDPC) or to stem infection (Su et al., 2006, McDonald & Peck, 2009). McDonald and Peck (2009) showed that with regular application of foliar fungicide on early sown field pea plants of cv. Alma, yield increased by 20%. They also calculated that with each 1% increase of stem infection, 1% of yield was lost, while stem girdling (coalesced lesions on stem) caused greater yield loss as each 1% stem girdled resulted in yield loss of 1.5%. In comparison, Bretag et al. (1995a) estimated that every 10% of stem area infection reduced field pea grain yield by 5-6%. In another study, total yield losses due to ascochyta blight were estimated to be 3.1 to 26.4% (Bretag, 1991). In a meta-analysis conducted by Salam et al. (2011c) over eight seasons during 1989-2003 in 13 farming locations in Western Australia, yield loss was estimated as 10.3% per unit of ascochyta blight severity, where severity was scaled from 0 to 5, being 0: no disease; 1: 1-20% stem area girdled; 2: 20-40% stem area girdled; 3: 40-60% stem area girdled; 4: 60-80% stem area girdled and 5: 80-100% stem area girdled. In some cases they observed that disease severity 5 resulted in more than 50 % yield loss.

The yield reduction due to disease can be estimated from studies which compare field pea yield with and without fungicide application; yield increases of 15-75% have been reported following application of foliar fungicides by various researchers (reviewed by Bretag (1991)). Although ascochyta blight incidence and severity differ according to genotypes, year, time of infection and regions, environmental moisture is the most crucial environmental factor affecting disease severity (Trapero-Casas & Kaiser, 1992, Roger et al., 1999a, b). Therefore, yield losses are greatest in a wet season on susceptible genotypes. In addition, the time of infection plays an important role in yield loss, for example, inoculation of plants at the flowering stage reduced yield by 24-34% compared to 19% when plants were inoculated at the seed fill stage (Xue et al., 1997).

1.5 Control of ascochyta blight of pea

1.5.1 Cultural practices

In Australia, cultural practices to manage ascochyta blight of field pea involve two strategies: (i) reducing the survival and spread of inoculum on crop stubble and in soil, and (ii) escaping the peak of inoculum from ascospores released after early autumn rain (McDonald & Peck, 2009, Salam et al., 2011b). Cultural methods, such as crop rotation involving periods of 3–6 years with non-host species and burying of field pea stubble by ploughing, are important methods to reduce the amount of ascochyta blight by minimising the amount of ascospores released from the stubble in Australian conditions (Bretag et al., 2006, Davidson & Kimber, 2007). Also, avoiding sowing field pea crops close to field pea stubble or early sown field pea crops and controlling self-sown volunteer peas in the field or at the border can reduce the risk of transmission of ascospores as primary inoculum to the new crop (Tivoli & Banniza, 2007, Hawthorne et al., 2012).

A delay in sowing is the method used to avoid the main release of ascospores of *D. pinodes* from field pea stubble (Peck et al., 2001, Zhang et al., 2005, McDonald & Peck, 2009, Yeatman et al., 2009, Salam et al., 2011a, Salam et al., 2011b, Salam et al., 2011c, Davidson et al., 2013). Because field pea stubble is the main source of primary inoculum of *D. pinodes*, and this inoculum is usually released with the autumn rains, delayed sowing is an effective method to avoid this inoculum and so reduce severity of ascochyta blight on young seedlings (Heenan, 1994, Bretag et al., 2000, Davidson & Ramsey, 2000, Peck et al., 2001, Bretag et al., 2006, Davidson & Kimber, 2007, Tivoli & Banniza, 2007, McDonald & Peck, 2009, Yeatman et al., 2009). There is evidence that ascochyta blight reaches a maximum severity in early sown crops and can be reduced to a minimum of nearly zero for late sown fields (Salam et al., 2011c).

In South Australian regions with less than 450 mm annual rainfall, field pea is usually sown in late May (Yeatman et al., 2009), coinciding with early season rainfall, which triggers the main release of *D. pinodes* ascospores (McDonald & Peck, 2009). This leads to more infection in early sown than late sown crops (Wroth & Khan, 1999, Davidson & Ramsey, 2000, Salam et al., 2011b, Hawthorne et al., 2012). McDonald and Peck (2009) reported that by delaying sowing of field pea seed by 3-4 weeks after the first autumn rain, ascochyta blight severity was reduced by 80% at 48 days after planting and grain yield increased by 6%, but with regular application of fungicides or the absence of disease on early sown crops, yield increased by 20%. As a consequence, it is recommended to farmers in these areas to delay planting by up to a month after autumn rains to minimise the risk of ascochyta blight (Peck et al., 2001, Yeatman et al., 2009). On the other hand, this delay in sowing is not without penalty to farmers, as it leads to

yield reduction in dry areas which experience moisture and heat stress at the end of the field pea season, particularly between flowering and harvesting time (McDonald & Peck, 2009, McMurray et al., 2011), especially in a dry finishing season (Salam et al., 2011c). South Australia had suffered drier and shorter growing seasons than average in the decade leading to 2010 and McMurray et al. (2011) confirmed that delayed sowing can be applied even under these conditions. They revised the optimum sowing period for field pea to 3 weeks after the first rains in autumn in areas with medium annual rainfall (429 mm) or medium-high (464 mm) and within one week of first autumn rains for low rainfall (329 mm) areas. McMurray et al. (2011) showed that 30% of yield could be lost due to ascochyta blight when they sowed with the first rains of autumn in a medium rainfall region. Moreover, it has been shown that more ascospores are produced on senescent plant materials in early sown field pea crops than later sown crops and this further increases disease intensity in spring (Davidson et al., 2013). This in turn may increase the risk of disease for the next season due to more infested stubble in the field.

As infested crop residue is important for survival of inoculum, burial or removal of residue is the best technique to minimise this source of inoculum for the next season (Carter & Moller, 1961, Galloway & McLeod, 2001, Gossen, 2001, Gossen & Miller, 2004, Zhang et al., 2005, Chilvers et al., 2007). Although zero-tillage or residue retention systems have been adopted in many countries in recent decades, reducing the burial of field pea stubble and its decomposition in soil, the effect of this practice on the epidemiology of ascochyta blight is still unclear (McDonald & Peck, 2009). For example, in one study in Saskatchewan, Canada, it was reported that a zero-tillage system did not increase field pea

mycosphaerella blight (synonym: ascochyta blight) and hence did not require application of fungicide unlike the conventional farming system (Bailey et al., 2000). Further research in the same region showed that severity of ascochyta blight of lentil was higher in a zero-tillage system than a conventional system where conditions were conducive to disease (Gossen & Derksen, 2003). Cutting and spreading pea stubble was recommended as an alternative method to burying stubble to promote decomposition and reduce soil erosion, but the plant bases remaining in the ground provide a source of inoculum (Hawthorne et al., 2012). Furthermore, grazing of field pea stubble or baling stubble in summer after harvesting of field pea are recommended to reduce stubble and consequently minimise the risk of ascochyta blight in following years (Hawthorne et al., 2012).

Salam et al. (2011b) developed the "G1 Blackspot Manager" model to predict the release of *D. pinodes* ascospore showers from field pea stubble at the beginning of the cropping season. This model forecasts the maturation of ascospores based on daily total rainfall and daily mean temperature. After pseudothecial maturity, release of ascospores can be predicted based on daily rainfall. Finally, based on ascospore release time and proximity to infested field pea stubble from last year, the optimum sowing time to avoid exposure of young seedlings to the peak ascospore release can be forecast to advise farmers to minimize the risk of ascochyta blight due to *D. pinodes* (Salam et al., 2011b). In the past, many growers have not applied basic recommendations for ascochyta blight management, such as delayed sowing or distance from infested field pea stubble (Davidson et al., 2013). Since the introduction of the online "G1 Blackspot Manager" tool in 2010, growers in South Australia, Western Australia and Victoria have increased adoption of management strategies (Department of

Agriculture and Food West Australia, 2010, Salam et al., 2011d). Early sowing can be advised using this model once sufficient rainfall has occurred before the desired sowing date (Salam et al., 2011b). However, this model is not applicable for the other pathogens of this disease complex which spread via pycnidiospores rather than ascospores. The second generation "Blackspot Manager" also considers abiotic stresses and advises growers when to sow to avoid a high risk of infection. Disease severity and potential yield loss can also be predicted by this model (Salam et al., 2011d, Department of Agriculture and Food West Australia, 2013).

Peck et al. (2001) showed that infection from soil infested with ascochyta blight pathogens decreased gradually over 3 years. In another study, soil-borne inoculum declined 15% per year over the first 4 years of an experiment in field conditions (McDonald & Peck, 2009). Based on these findings, soil inoculum may be reduced by 90% in 6 years; therefore a 6-year rotation with non-host crops was recommended to avoid soil-borne inoculum (McDonald & Peck, 2009). Hawthorne et al. (2012) stated that in soils with a high level of ascochyta blight pathogens, a 5-year break may not be enough time to reduce the soil-borne inoculum to a level where ascochyta blight is a low risk. However, *P. koolunga* has been detected by DNA test from field soil 4 years after the last field pea in South Australia (Davidson et al., 2011), the survival structures of this fungus in the soil has not yet been investigated. Hawthorne et al. (2012) recommended that growers in South Australia assess the risk of soil-borne ascochyta blight pathogens using Predicta^{TM}B (Ophel-Keller et al., 2008), which is a DNA-based soil testing service to identify fields with a high level of these pathogens in the soil, even if the previous crops were not field pea.

1.5.2 Chemical control; seed treatment and foliar fungicides

It has been reported that seedling emergence rate and yield were negatively correlated with the percentage of seeds infected by *D. pinodes* under field conditions (Xue, 2000), therefore, treatment of seed with fungicides is one way to reduce primary inoculum and seed to seedling transmission of seed-borne ascochyta blight. Kharbanda and Bernier (1979) stated that in laboratory assays, thiabendazole and benomyl effectively controlled disease transmission in seeds infected with *A. fabae*. Similarly, Ali et al. (1982) demonstrated that both benomyl and thiram significantly controlled seed-borne *M. phaseolina*, *P. medicaginias* and *A. pinodes in vitro* and a mixture of these two chemicals gave complete control of seed to seedling transmission and did not affect seed germination. Also, Xue (2000) showed that thiram increased seedling emergence from seed infected with *D. pinodes*. A survey conducted by Peck and McDonald (2001) revealed that 90% of South Australian farmers did not use fungicides for seed treatment or as post-emergent treatment to control ascochyta blight and Bretag et al. (2006) stated that application of seed dressing was not economic due to high cost of fungicides and low price of field pea in Australia. In comparison, Hawthorne et al. (2012) declared that this treatment can be recommended for some regions based on the disease risk, although they alerted readers to the negative effect of fungicidal seed dressings on rhizobia especially in acidic soils. Therefore, this practice has been suggested for fields with high disease risk, particularly where long rotation with other crops to reduce disease risk is not applicable (Hawthorne et al., 2010). Fungicides with the active ingredients thiram and thiabendazole, such as P-Pickel T®, Fairgro® and Reaper® TT, at the rate of

200 ml per 100 kg seed, have been recommended to control ascochyta blight seed infection as well as seedling root rot (Hawthorne et al., 2010, 2012).

Many foliar fungicides have been applied to control ascochyta blight of field pea including, benomyl, thiabendazole, carbendazim, metiram and chlorothalonil, which may be protective and eradicative and also have better residual activity than older fungicides such as Bordeaux mixture, copper oxychloride and lime-sulfur (Warkentin et al., 1996, 2000, Bretag et al., 2006, Hawthorne et al., 2010, Salam et al., 2011c). Ascochyta blight can be effectively controlled with multiple sprays of foliar fungicides, but this is not economic, particularly when yields are not high (Bretag et al., 2006) or in early sown fields with high disease pressure (Salam et al., 2011c). In South Australia, Davidson et al. (2004) observed a 12.6% yield increase in sprayed field pea crops (3.57 t/ha) compared with unsprayed crops (3.17 t/ha) and suggested that the application of foliar fungicides can be economic if the grain yield is expected to be higher than 2.5 t ha^{-1} (Davidson, 2012). McMurray et al. (2011) reported that applying P-Pickel T® as a seed dressing plus fortnightly sprays of chlorothalonil increased yield by 55% in early sown pea plots compared to a 30% increase in late sown plots in the medium rainfall region. However this strategy was considered uneconomic, whereas with the economic strategy of a seed dressing plus two sprays of mancozeb they observed only 12% yield increase in early sown (with autumn rains) and mid-sown (3 weeks after autumn rains) plots. They also concluded that application of fungicides would be uneconomic if the field pea grain price fell lower than AUD $200 per tonne, but in the last two years grain prices have increased to above AUD $300 per tonne.

On the other hand, in France, application of fungicides to spring sown field pea crops is economic due to a greater potential yield and a higher disease

potential as the results of superior moisture levels. The first application is recommended at the beginning of flowering followed by a second spray 15 days later and, finally, a third application after another 10 days (Su et al., 2006, Schoeny et al., 2007). Therefore, strategic spraying of appropriate fungicides and a limited number of sprays in Australian conditions (McMurray et al., 2011), as well as using risk models to predict the best time of application, can benefit farmers when combined with application of agronomic practices to minimise disease pressure.

1.5.3 Resistance of field pea genotypes

The development of ascochyta blight resistant field pea genotypes would be the most effective and durable strategy to control this disease, but so far resistance has remained elusive (Bretag et al., 2001, 2006, Tivoli et al., 2006, Davidson & Kimber, 2007). By growing disease resistant plants, farmers would not only save money on fungicides and their application, but also such genotypes are safer for the environment and decrease the danger of emergence of resistance to fungicides. Unfortunately, no complete resistance to ascochyta blight pathogens has been found, even though a great deal of effort has been expended on screening field pea lines for resistance to these fungi (Bretag et al., 2006, Fondevilla et al., 2011). For example, Kraft et al. (1998) could not find any field pea line resistant to *D. pinodes* after screening 2,936 accessions of *Pisum sativum* in field conditions, and Xue and Warkentin (2001) reported that, after screening 335 field pea lines, only seven lines showed partial resistance. Also, Jha et al. (2013) could not find any genotype resistant to *D. pinodes* after assessing 169 genotypes under field conditions in Canada from diverse pea germplasm sourced from Eastern and Western Europe, Australia and Canada. It has been reported that not only is the

resistance to each of *D. pinodes*, *P. medicaginis* var. *pinodella* and *A. pisi* under separate genetic control, but also, for each of these pathogens, the resistance within each organ, such as stem, foliar part, seed and root, is also under separate genetic control (Sakar et al., 1982, Bretag et al., 2006).

Resistance of field pea plants to ascochyta blight is, at best, partial. Also assessing field pea genotypes in field conditions is difficult and depends on environmental conditions which affect disease expression and often results are not consistent (Porta-Puglia et al., 1993). Therefore, a number of methods using a controlled environment have been developed. A detached leaf method whereby leaves are floated on water in Petri dishes and droplets of spore suspension are deposited on the upper surface of the leaf was reported as reliable and requires little space. The reaction can be assessed in 7 days (Onfroy et al., 2007, Richardson et al., 2009). Richardson et al (2009) observed a significant interaction between *D. pinodes*, *P. medicaginis* var. *pinodella*, *A. pisi* and *P. koolunga* and field pea genotypes for disease symptoms and severity using the detached leaf method. Spraying pycnidiospore suspensions on whole plants in pots in a greenhouse and scoring disease severity is another method to evaluate the reaction of field pea lines to those pathogens (Onfroy et al., 1999, Prioul et al., 2003, Onfroy et al., 2007).

Until now, there has been no specific study done on the evaluation of current and new field pea genotypes to a range of isolates of *P. koolunga*. Therefore, there is a need to assess these genotypes of field pea for response to inoculation with *P. koolunga* isolates.

1.6 Summary and aims of research

Ascochyta blight of field pea reduces both production and yield of pea around the world, including Australia and South Australia (Bretag & Ramsey, 2001) and is caused by up to five fungal pathogens, of which *P. koolunga* was recently described (Davidson et al., 2009a).

As *P. koolunga* has only recently been described, there is little published information on aspects of biology and epidemiology of this pathogen, such as survival, seed infection and resistance of field pea genotypes to this pathogen. Also, the teleomorph of this fungus is still unknown.

The aims of the research presented in this thesis were to understand the survival of *P. koolunga* on stubble, in soil and seed and investigate the possibility of formation of its teleomorph. Therefore the objectives of this project were to: (i) determine the role of field pea stubble and pseudosclerotia in survival of *P. koolunga*; (ii) explore whether this pathogen is seed-borne or not and investigate the potential role of seed-borne infection in the disease cycle of *P. koolunga*; (iii) study whether *P. koolunga* can produce sexual fruiting bodies or not and, if so, determine in what conditions; (iv) assess reaction of existing pea genotypes to *P. koolunga.*

1.7 Linking statement

This research thesis has six chapters, including three research papers; Chapters 2, 3 and 4; which have been prepared for publication in peer reviewed journals.

The first chapter presents an introduction of ascochyta blight of field pea, epidemiology and control of this disease in Australia and around the world. The summary and the aims of this research are presented at the end of this chapter.

Research on survival and recovery of *P. koolunga* from field pea stubble placed on the soil surface or buried in the soil and longevity of pseudosclerotia in the soil is presented in Chapter 2.

Chapter 3 consists of research on seed-borne infection of this fungus, its survival, transmission to seedlings at a range of 8-20° C soil temperatures and control of *P. koolunga* in seed. In addition, the reaction of field pea genotypes to this pathogen in controlled condition is presented in this chapter.

Chapter 4 describes the appearance of sectors of atypical colonies of *P. koolunga* on culture media or isolates from field pea seed. The comparison of these cultures with typical isolates of *P. koolunga* in terms of production of pycnidia and pycnidiospores, colony growth on culture media and virulence on field pea seedlings is presented in this chapter.

Research on methods to initiate formation of the teleomorph of *P. koolunga* in controlled conditions and explanation of the possible reasons for failure of all attempts to produce pseudothecia of this fungus are presented in Chapter 5.

Chapter 6 provides a general discussion. Results of research presented in chapter 2 to chapter 5 are discussed briefly. Subsequently, the importance and

possible application of these results in management of ascochyta blight caused by *P. koolunga* on field pea plants in Australian conditions are presented.

Chapter 2

Survival of *Phoma koolunga*, a causal agent of ascochyta blight, on field pea stubble or by pseudosclerotia in soil

Manuscript prepared for submission to Plant Pathology

Survival of *Phoma koolunga*, a causal agent of ascochyta blight, on field pea stubble or by pseudosclerotia in soil

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Abstract

Phoma koolunga is a recently recognised pathogen in the ascochyta blight complex of field pea (*Pisum sativum*). Unlike the other three ascochyta blight pathogens, survival of *P. koolunga* is poorly understood. Survival of this fungus was examined on field pea stubble and pseudosclerotia on the surface of, and buried in, field soil. Pseudosclerotia were formed in plates containing potato dextrose agar (PDA) mixed with sand or amended with fluorocytocin. After one month, *P. koolunga* was recovered on amended PDA from 93% of stubble sections retrieved from the soil surface, 36% of buried stubble sections and 100% of pseudosclerotia buried in field soil, pasteurised or not. Frequency of *P. koolunga* recovered decreased over time and the fungus was not recovered from stubble on the soil surface at month 15, whereas it was not recovered from stubble buried in soil at months 11 and later or from pseudosclerotia buried for 18 months. In a pot bioassay, most ascochyta blight lesions developed on plants inoculated with stubble retrieved from the soil surface after one month. Infectivity of the inoculum

decreased over time. Disease on plants inoculated with stubble that had been buried or left on the soil surface for up to 6 and 5 months, respectively, and pseudosclerotia retrieved at 14 months and later from field soil did not differ from the uninoculated control. These results suggest that field pea stubble may play a role in survival of *P. koolunga*, especially if it remains on the soil surface. In addition, pseudosclerotia are able to persist in soil and infect field pea plants into the next season.

Key words: Infested stubble, stubble burial, *Pisum sativum*, infectivity

Introduction

Ascochyta blight is a devastating foliar disease of field pea (*Pisum sativum* L.) in Australia and around the world (Bretag & Ramsey, 2001). It is considered the most important disease of field pea in Australia, responsible for 15% production losses annually and causing up to 75% yield loss in individual crops (Bretag et al., 1995; Salam et al., 2011a; Davidson, 2012). This disease is usually referred to as the ascochyta blight complex, because it is caused by a number of closely related fungal species which exist independently of each other. Until 2009, three species were recognised as causal agents of ascochyta blight, i.e. *Didymella pinodes* (syn. *Mycosphaerella pinodes*), *Phoma medicaginis* var. *pinodella* and *Ascochyta pisi*. Davidson et al. (2009a) characterised *Phoma koolunga* as a fourth fungal species which can cause ascochyta blight on field pea.

The application of foliar fungicides to control ascochyta blight on field pea is usually uneconomic (Warkentin et al., 2000; McMurray et al., 2011) and resistant pea genotypes are not available (Bretag et al., 2006). Therefore, disease control relies on cultural methods. Burying infested field pea stubble, rotations after field pea crops and a delay in sowing seed to avoid the peak release of ascospores from infested stubble are

68 considered the most important practical methods to manage this disease (Bretag et al.,
69 2006; McDonald & Peck, 2009; Salam et al., 2011b; Davidson, 2012).

70 Incidence and severity of ascochyta blight increase when field pea crops are sown
71 in the vicinity of field pea stubble infested with *D. pinodes* (Davidson & Ramsey, 2000;
72 Galloway & McLeod, 2001; Bretag et al., 2006; Davidson et al., 2013). This is likely
73 due to production of survival structures of the causal agents on the stubble (Dickinson
74 & Sheridan, 1968; Zhang et al., 2005; Bretag et al., 2006; McDonald & Peck, 2009;
75 Davidson et al., 2013). The major pathogen of the ascochyta blight complex, *D.*
76 *pinodes*, can survive from 4 to 18 months as pseudothecia or sclerotia on field pea
77 stubble, or as chlamydospores in infested soil for at least 12 months (Zhang et al., 2005;
78 Bretag et al., 2006). Moreover, Davidson et al. (1999) reported that after 4 months,
79 stubble from ascochyta blight-affected field peas incubated on the soil surface or
80 underground was not infectious. McDonald and Peck (2009) showed that the amount of
81 *D. pinodes* inoculum on field pea stubble was high the year after a field pea crop and
82 declined rapidly after one season. Recently, Davidson et al. (2011) showed that in a
83 bioassay test the severity of ascochyta blight symptoms, including stem lesions and leaf
84 spots, was positively correlated to quantity of soil borne inoculum of these pathogens,
85 including *P. koolunga*.

86 The survival of *D. pinodes*, *P. medicaginis* var. *pinodella* and *A. pisi* on field pea
87 residues is well-documented, however, information about *P. koolunga* is lacking.
88 Although Davidson et al. (2009a) reported that pseudosclerotia of *P. koolunga* may be
89 present in culture, pseudosclerotia have not been described in any detail and their role in
90 the survival of *P. koolunga* is completely unknown. The objectives of this study were to
91 examine the survival of *P. koolunga* over time by burying infested field pea stubble in

soil or placing it on the soil surface and to study the production of pseudosclerotia in culture media and their survival in soil.

Materials and methods

Stubble and pseudosclerotia – production and burial

Field pea stubble that was heavily infested with *P. koolunga* was collected from experimental potted plants at Waite Campus, South Australia (SA) in November 2011. These plants had been artificially inoculated 3 weeks post-sowing with a mixed pycnidiospore suspension (5×10^5 spore/ml) from four isolates of *P. koolunga,* 139/03, 142/03, 81/06 and FT07026, until runoff. The inoculation was repeated three times at 6-day intervals. The harvested stubble was stored at 4°C until used. Stubble was placed in bags and buried using methods adapted from Naseri et al. (2008). Briefly, basal parts of the stems from the crown to 40 cm above were cut into 10-cm pieces and 104 lots, each comprising 15 stem pieces, were weighed individually and placed in 15 cm^2 plastic mesh bags (mesh pore size 1 mm^2). Each bag was placed on, or buried 5-10 cm below, the soil surface in 20-L pots outdoors at the Waite Campus in February 2012. This soil was collected from a field with no history of field pea at the Lenswood Agricultural Centre, approximately 30 km east of Adelaide, SA and subjected to DNA analysis by the Root Disease Testing Service (RDTS) at the South Australian Research and Development Institute (Davidson et al., 2009a) to confirm it was free from *P. koolunga*. The pH and electrical conductivity (EC) of field soil were measured using a pH meter and a conductivity meter and the soil texture was predicted by MIR spectroscopy (Janik et al., 1998). The daily rainfall and average air temperatures for Glen Osmond, 2.8 km from the Waite Campus, were obtained from the Australian Bureau of Meteorology.

To initiate formation of pseudosclerotia, twice-autoclaved river sand (3-4 g, Sloan's Sands Pty Ltd) was added to molten potato dextrose agar (PDA) in Petri dishes

(9 cm in diameter) and, when solidified, a 2-mm plug of *P. koolunga* (isolate FT07026) was placed in the middle of the plate. The plates were incubated at 22°C in the dark for 4 weeks. Pseudosclerotial masses produced in the plates were cut into 2 cm^2 and mixed with 20 g of sterilized sand (Coley-Smith, 1985) in nylon mesh bags (8 × 8 cm, 20 µm pores; Schweizer Seidengaz-fabrik AG, Thal, Switzerland) (Probst, 2011); the edges were sealed and bags were buried in pasteurised or non-pasteurised field soil in 2-L pots in March 2012.

Recovery of *P. koolunga* and associated mycobiota from stubble in or on soil, or from pseudosclerotia

Prior to burying stubble in soil, 15 pieces of randomly selected field pea stubble were tested for presence of *P. koolunga* using the methods described below, which were adapted from Naseri et al. (2008). From March 2012 four replicate bags from each treatment, on and in soil, were retrieved monthly for 12 months and a final set was removed in month 15, May 2013. Briefly, the stubble from each bag was rinsed, air-dried and weighed. Representative pieces of stubble were examined for the presence of pycnidia and pseudosclerotial masses. From each of five randomly selected pieces per bag, four 0.5-cm segments were excised, from each end and from the middle. These stem segments then were immersed in 0.5% sodium hypochlorite for 3 min in the first 6 months and thereafter 1 min due to the fragility of the stubble, followed by rinsing in sterile distilled water and drying on sterile filter paper for 2 h. The stem segments were plated onto semi-selective agar medium, developed in preliminary experiments, which comprised PDA amended with 45 mgl^{-1} fluorocytocin and 100 mgl^{-1} streptomycin. Fungal growth was recorded during 10 days of incubation at 22°C under 12 h fluorescent and near ultraviolet light and 12 h dark, after which colonies were transferred to PDA in the same incubation conditions.

The percent isolation frequency from each bag was recorded as the number of small stem sections that yielded *P. koolunga* divided by 20. The same formula was also applied for other fungi most commonly recovered from stubble from July 2012 to May 2013. Fungi were identified to the genus on the basis of morphological characteristics (Barron, 1968; Ellis, 1971; Ellis, 1976; Domsch et al., 1980; Barnett & Hunter, 1998; Watanabe, 2010).

From April 2012, four replicate bags of pseudosclerotia from non-pasteurised and pasteurised soil were retrieved monthly for 11 months, again in month 14 and a final set was removed in month 18, in September 2013. At each sampling time, four bags from each treatment were randomly removed and the pseudosclerotia were shaken in five changes of sterile water (Dickinson & Sheridan, 1968) amended with streptomycin (100 mgl^{-1}), then dried on sterile filter paper. The pseudosclerotia masses were cut to small fragments of approximately 0.3-0.5 mm^2, using a sterile scalpel and then were transferred to PDA, 10 pieces per plate. These plates were incubated in the conditions described above for up to 10 days and viability of pseudosclerotia was assessed as the percentage of pseudosclerotial masses that germinated.

Effect of burial on infectivity of the inoculum of *P. koolunga*

The infectivity of inoculum of *P. koolunga* on retrieved stubble over time was tested in a pot bioassay using methods adapted from Davidson and Krysinska-Kaczmarek (2003). Each month, five seeds of field pea cv. Kaspa were sown in each MK12 pot (7 × 8.5 × 5.5 cm) containing University of California (UC) potting soil (Baker, 1957). Four replicate pots were used for each treatment, plants were grown in a growth room at 14-16°C with 12 h light/12 h dark and watered as required. Plants at four nodes growth stage were sprinkled with 0.5 g milled field pea stubble, maintained in a humidity chamber for 48 h, and then transferred to a growth room and watered as before. For

control plants, sterile water was used instead of milled stubble. The number of ascochyta blight lesions on leaves and stems on each plant was counted after 10 days. Representative diseased leaves were examined for presence of *P. koolunga* by isolation on PDA plates.

The infectivity of pseudosclerotia retrieved from soil was assessed using a method adapted from Yaqub and Shahzad (2005) and Coley-Smith et al. (1990) at 1, 4, 7, 10, 14 and 18 months post-burial. In brief, plugs of *P. koolunga* containing pseudosclerotia (3 cm^2) were dried, crushed and spread on sterilized UC soil in pots (7 × 6 × 6 cm), then covered with a further 4 cm layer of sterilized UC soil. Ten seeds of field pea cv. Kaspa were sown in each pot at a depth of 2.5 cm. The pots were kept in the growth room in conditions described above. After 21 days, roots were washed and assessed for symptoms and isolation of fungus.

Statistical analysis

Analysis of variance (ANOVA) were applied to the data for isolation frequency of *P. koolunga* or other fungi from stubble, recovery from pseudosclerotia and infectivity tests using GenStat 15^{th} edition SP2. To test trends in isolation frequencies of fungi from stubble for sampling month and depth, linear and quadratic analyses were conducted. Tukey's honestly significant test at 95 % confidence level was applied to compare means in each experiment.

Results

Stubble burial

The field soil was determined to be sandy-loam with pH 5.38 and EC of 113.7 mg L^{-1}. The monthly average air temperature at Waite Campus, South Australia for the duration of the experiment is shown in Fig. 1. The daily temperature during this research

fluctuated between 10.8°C in June 2012 (winter) and 40.5°C in January 2013 (summer). Maximum and minimum monthly rainfalls were recorded in June 2012 (130 mm) and March 2013 (11.2 mm), respectively (Fig. 1). In overall, the climate conditions during this research were similar to the average of the last 10 years. For example, the annual rainfall at Waite Campus for 2012 was 567 mm compared to the average of 626 mm (Bureau of Meteorology of Australia, 2013).

After one month, the weight decrease was 4% for both stubble placed on, or buried in soil, but after 2 months, the stubble buried in soil lost significantly more weight (9%) than stubble placed on the soil surface (5%) (Table 1). This weight reduction continued over time for stubble both on soil and in soil, however at a greater rate for buried stubble; at the end of the experiment (after 15 months), stubble weight decrease was 28 and 53% for soil surface and buried, respectively. Decomposition of stubble buried in soil was first seen after 4 months and after 8 months some stems were broken in the bags retrieved from soil. The stubble placed on the soil surface did not show signs of decomposition until 12 months, when pith was decayed. Pycnidia were observed on most pieces of stubble before placement and after retrieval, but pseudosclerotia were not found.

P. koolunga and other fungi isolated from stubble

In February 2012, prior to placing or burying field pea stubble on or in soil, *P. koolunga* was isolated from 92% of stubble pieces. After one month of incubation, the same isolation frequency was recorded for stubble placed on the soil surface, but the isolation frequency for buried stubble decreased to 36% (Fig. 2). Isolation decreased over time for both treatments, except for April, June and September 2012 for stubble in soil, and December 2012 and February 2013 for stubble on the soil surface. In addition, Tukey's test ($P < 0.05$) showed that survival of *P. koolunga* on stubble buried in soil for months

9 and 10 was not significantly different, but it was statistically different from month 11 and thereafter, when no isolation was recorded. However, the fungus was isolated from 2.5% and 5% of stubble pieces retrieved from the soil surface in January and February 2013, respectively, but it was not recovered from the last set retrieved in May 2013.

Fusarium spp. were the fungi most frequently isolated from stubble buried in soil over time, ranging from 51% in July 2012 to 19% in February 2013 (Table 2). These fungi were recovered at a maximum frequency of 32% of stubble pieces placed on the soil surface after 10 months and a minimum of 5% in July 2012 and May 2013. The second fungus most frequently isolated from buried stubble was *Stachybotrys chartarum* with an average of 25% in 9 months of sampling from July 2012 to May 2013; however, it could not be detected in surface-placed stubble in five of the nine sampling periods. The mean isolation frequency of *Trichoderma* spp. was 12%, fluctuating between 0% in July and 21% in August 2012 for buried stubble and between 0% in December 2012 and February 2013 to 11% in September 2012 for stubble placed on the soil surface. *Gliocladium* spp. were isolated from buried stubble in each of the 9 months of recording, most frequently at 11% in February 2013. This genus was recovered from stubble placed on the soil surface in only four of the nine sampling periods and then only at a frequency of 4% or less.

Recovery of *P. koolunga* from pseudosclerotia

P. koolunga was recovered from 100% of pseudosclerotial masses buried in pasteurised and non-pasteurised soil until the fifth and sixth month post-burial, respectively (Fig. 3). Then the percentage recovery decreased in a linear trend each month for the remainder of the experiment, with a lower isolation frequency ($P < 0.05$) in non-pasteurised soil than pasteurised soil, dropping to 3% and 9%, respectively, in May 2013 after 14

240 months. The fungus did not grow from pseudosclerotial masses onto culture medium
241 after 18 months of burial in either pasteurised or non- pasteurised soil.

242 **Effect of burial on infectivity of *P. koolunga* infested field pea stubble**

243 Most ascochyta blight lesions developed on leaves and stems of plants inoculated with
244 stubble retrieved after one month from the soil surface or buried in soil, *viz*. 26 and 9
245 lesions per plant, respectively (Fig. 4). In general, the infectivity of the inoculum of the
246 fungus on stubble buried in or placed on soil decreased over time. A sharp decrease in
247 infectivity of the inoculum of the fungus on surface-placed stubble was recorded
248 between June and July 2012, 4 and 5 months after placement, to the level which was not
249 significantly different from the water control. Disease on plants inoculated with stubble
250 retrieved 6 months and more after burial did not differ from the water control. This was
251 also the case for stubble 3 months after burial. Infectivity of the inoculum of the fungus
252 placed on soil surface at 5 months and later was not significantly different to water
253 control ($P < 0.05$). No lesions were observed on control plants inoculated with sterile
254 water throughout the experiment.

255 Plants grown from seeds sown in soil inoculated with pseudosclerotia of *P. koolunga*
256 showed symptoms as limited (< 4mm in length) or developed (4-11 mm) necrotic
257 lesions on roots and epicotyls, 3 weeks after sowing. *P. koolunga* was isolated from all
258 representative lesions excised from seedlings roots. Root symptoms were most
259 frequently observed on plants grown for 21 days in soil mixed with pseudosclerotia that
260 had been buried for one month, with 82% and 80% disease incidence in non-
261 pasteurised and pasteurised soil, respectively (Fig. 5). Disease incidence on the roots
262 decreased as duration of burial of pseudoscerotia in soil increased, dropping to 20% and
263 5% after 10 and 14 months for non-pasteurised and pasteurised soil, respectively. No

root lesions were seen on seedlings in soil inoculated with pseudosclerotia retrieved from non- pasteurised or pasteurised soil after 14 and 18 months, respectively.

Discussion

Survival of *P. koolunga* was greater on field pea stubble left on soil than buried in soil; viability of the fungus decreased at different rates over time such that it was not recovered after 11 and 15 months from stubble buried in top of the soil or placed on soil, respectively. The stubble decomposed more rapidly in soil than on the soil surface. Infectivity of inoculum of *P. koolunga* also in stubble decreased over time. Pseudosclerotia remained viable in pasteurised soil longer than in field soil, and infectivity was lost earlier than viability.

The infectivity of inoculum of *P. koolunga* on stubble placed on or buried in soil decreased faster than did viability. The mean number of lesions per plant inoculated with stubble after 5 months placement on soil surface or 6 month burial decreased to a level equivalent to the water control. In a similar study, Zhang et al. (2005) reported that the ability of *D. pinodes* to produce ascospores on field pea leaf or stem residue placed on or buried 5-10 cm deep in soil decreased to an unmeasurable level after 9 months. They stated that disease severity resulting from applying washings from buried stubble to bioassay plants was high for the first 2-4 months. The results of the current study were also in agreement with Davidson et al. (1999), in that field pea stubble infested with *D. pinodes* and *P. medicaginis* var. *pinodella* remained highly infectious for the first 4 months post-burial in soil, but did not initiate severe disease thereafter. In comparison, *P. koolunga* survived better in stubble left on the soil surface than in stubble buried in soil, which appeared to be due to lesser decomposition of the stubble on the soil surface. For example, stubble weight decreased by 16% after 4 months in soil, but after 8 months on the soil surface. Naseri et al. (2008) reported that

289 *Leptosphaeria maculans* on stubble remained pathogenic and caused phoma leaf spot on
290 oilseed rape seedlings until 9 months post-burial in soil or sand, similar to the duration
291 of time was recorded for *P. koolunga* on field pea stubble buried in soil in this research.
292 Environmental conditions such as rainfall and temperature affect the survival and
293 growth of fungi (Baird et al., 1999; Baird et al., 2003) and Zhang et al. (2005) explained
294 that less decomposition was observed on surface-incubated stubble in Canada as it was
295 drier than buried stubble, especially in winter and spring. The same is likely to be the
296 case in South Australia.

297 *Fusarium, Gliocladium, Trichoderma,* and *Stachybotrys* spp. were isolated
298 frequently from field pea stubble. These fungi have been reported to antagonise other
299 fungi and also are able to decompose plant residue (Domsch et al., 1980). These fungi
300 were isolated more frequently from buried stubble than stubble left on the soil surface,
301 perhaps due to greater moisture content and better physical protection for microbes in
302 soil than on the soil surface (Van Veen & Kuikman, 1990). Burial of stubble may hasten
303 decomposition and promote colonisation of stubble by antagonistic microorganisms.
304 Carbon released from decomposed residue promotes soil microbial activity and so
305 increases pathogen degradation (Raaijmakers et al., 2009). *Trichoderma* spp., which
306 were isolated from almost all stubble retrieved after burial in soil in this study, are well
307 known for rapidly colonizing plant residue in contact with soil as well as parasitising
308 plant pathogens, such as *Rhizoctonia, Pythium, Fusarium* spp., in the field and
309 *Mycosphaerella phaseolina in vitro* (Harman, 2000; Baird et al., 2003). Baird et al.
310 (2003) isolated *Trichoderma* spp. from root segments of soybean in soil and stated that
311 *Trichoderma* spp. with the ability to parasitise pathogens and degrade plant cellulose
312 may reduce the survival of *M. phaseolina*. The same explanation may apply to *P.*
313 *koolunga* on field pea stubble buried in soil and this merits further investigation.

Davidson et al. (2009a) mentioned that pseudosclerotia of *P. koolunga* may form on radiating lines of pale yellow-brown to dark brown mycelium on artificial media, but no details of pseudosclerotia were provided. In this study, pseudosclerotia were formed on PDA that contained grains of sand while on PDA without sand only mycelial growth was observed. In addition, in antifungal sensitivity tests, large numbers of pseudosclerotia of *P. koolunga* were formed when 100 mgl^{-1} fluorocytocin was included in the medium, although at 45 mgl^{-1} fluorocytocin the fungus continued vegetative growth and produced only pycnidia and pycnidiospores. It seems that nutritional or mechanical factors or antifungal chemicals influence the formation of pseudosclerotia by *P. koolunga*, as has been reported for other fungi (Townsend, 1957; Chet & Henis, 1975; Camyon & Gerhardson, 1997). For example, Henis et al. (1965) reported that sclerotia of *Sclerotium rolfsii* usually formed on culture media in Petri dishes when the mycelium reached the edge and linear growth was restricted. It is possible that contact with the hard surface of the grains of sand elicited a similar response in *P. koolunga*. Furthermore, antifungal antibiotics such as bacitracin, trichomycin and griseofulvin induced formation of sclerotia by *S. rolfsi* (Chet & Henis, 1975) and a similar effect on formation of pseudosclerotia of *P. koolunga* was seen when fluorocytocin was incorporated into PDA. Pseudosclerotia in the current study were dark brown, firm, irregular in shape and 105 to 410 µm in diameter. They mostly consisted of dark brown mycelia with thick-walled cells (15.1-20.2 µm in diameter). The pseudosclerotia of *P. koolunga* were morphologically similar to those reported for the potato gangrene pathogen, *Phoma foveata*, (Camyon & Gerhardson, 1997) and for *P. chrysanthemicola* (Dorenbosch, 1970). Pseudosclerotia of *P. koolunga* were usually initiated at 2 weeks and developed in 4 weeks on the above-mentioned media and appeared to be formed as

338 survival structures in adverse conditions. Also they survived in soil and remained
339 pathogenic for longer than other structures of *P. koolunga*, such as pycnidia on stubble.
340 Although 100% of pseudosclerotia buried in soil were viable on PDA plates for
341 up to 5 months, thereafter a reduction was observed; more so for pseudosclerotia in non-
342 pasteurised soil than pasteurised soil. In agreement with these results, the viability of
343 sclerotia of *Colletotrichum coccodes* and *Phoma liguilicola* buried in soil was greater in
344 sterile moist garden soil than non-sterile soil after 6 and 2 months, respectively
345 (Blakeman & Hornby, 1966). The same trend was observed for sclerotia of *C. coccodes*
346 buried in sterile, dry garden soil compared to non-sterile soil for 12 months. It appears
347 that soil microorganisms are responsible for greater decomposition and lysis of buried
348 pseudoscleotia in non-sterile soil than sterile soil (Blakeman & Hornby, 1966).
349 In the current study, 96% of pseudosclerotial masses of *P. koolunga* buried in
350 non- pasteurised soil remained viable after 6 months. Dickinson and Sheridan (1968)
351 also reported that after burial of sclerotia of *D. pinodes* in soil for 4 months, 90%
352 germinated and they attributed this to the persistent nature of sclerotia. Results from the
353 current study suggest that pseudosclerotia of *P. koolunga* buried in field soil in the
354 absence of host plants remain pathogenic for at least 10 months. Ten months is
355 sufficient for pseudosclerotia to infect the root system of the next season's field pea
356 plants and possibly also alternative hosts. In comparison, infectivity of sclerotia of *P.*
357 *liguilicola* on chrysanthemum cuttings was lost after burial for 2 months in non-sterile
358 compost, whereas 7% of tomato seedlings inoculated with sclerotia of *C. coccodes*
359 retrieved from natural soil after 20 months were infected (Blakeman & Hornby, 1966).
360 It seems that the longevity and infectivity of persistent structures such as
361 pseudosclerotia or sclerotia of different fungi varies and these differences have been
362 attributed to the type of morphological development *viz.* loose, terminal or strand

(Blakeman & Hornby, 1966). The type of pseudosclerotia of *P. koolunga* has not yet been determined. *P. koolunga* survived as pseudosclerotia in sterilized soil for more than 14 months, which is longer than survival of pycnidia or mycelia on stubble in soil up to 11 months.

Davidson et al. (2011) reported that DNA of *P. koolunga* could be detected in field soil 4 years after a field pea crop was grown and predicted that the level of soil borne inoculum could initiate an ascochyta blight epidemic, but they did not assess infectivity, nor indicate the means by which the fungus might survive. The current study suggests that *P. koolunga* may not have been viable 4 years after the last field pea crop.

Although this research showed that infested field pea stubble left on the soil surface did not initiate severe disease after 5 months, this 5 month window could allow early sown or volunteer field pea crops or alternative hosts to be infected by *P. koolunga*. Therefore, burying stubble would shorten survival of the pathogen and limit release and dissemination of conidia (Davidson et al., 2013). On the other hand, burying plant debris is not compatible with zero tillage or residue retention farming systems which have been adopted in recent decades. As a consequence, the general recommendation of delayed sowing or distance from field pea stubble infested with *D. pinodes* (Peck et al., 2001; Davidson et al., 2013) can be applied to *P. koolunga* as well. In addition, other legume crops may be alternative hosts of this fungus (Ali & Dennis, 1992; Bretag et al., 2006; Davidson et al., 2009b). Possible alternative hosts of *P. koolunga* might be infected not only by pycnidiospores and mycelia, but also by pseudosclerotia via root systems if sown in the same field in the next season. Therefore, research on host range of *P. koolunga* is required. If any other crop plants become infected by survival structures of this fungus, their use in rotation with field pea or even in the vicinity of infested stubble from the previous year would need to be reviewed.

So far, no sexual stage has been reported for *P. koolunga* and this study showed that pycnidia or mycelia on plant residue are not likely to survive for long periods of time. Thus, pseudosclerotia may be responsible for longer survival than other structures and may pose a greater risk to the next season's field pea crops than infested stubble buried in soil. The question remains as to whether or not *P. koolunga* can produce pseudosclerotia in natural conditions. Investigation of the formation of these structures on plant material or in soil and understanding their importance in survival of the fungus and possible role in the epidemiology of the pathogen in field pea crops is recommended.

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529 survival of *Mycosphaerella pinodes* in Manitoba. *Canadian Journal of Plant*
530 *Pathology* **27**, 132-36.

531

532 Table 1 Mean percentage decrease in weight of field pea stubble buried in or placed on soil in February 2012 over 15 months

	Sampling month												
	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	May
On soil surface[a]	4.53	5.63	7.87	9.44	11.54	10.79	13.53	16.16	19.22	21.35	24.17	24.86	27.86
In soil	4.64	9.27	13.19	16.11	18.37	19.45	20.75	22.87	26.88	30.11	32.32	36.59	52.76

533 [a]Mean percentage decrease of stubble weight in four replicate bags; LSD = 1.55 (whole data set) , $P < 0.01$

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544 Table 2 Mean isolation frequency (%) of common fungi from field pea stubble placed on or buried in soil from July 2012 to May 2013

Fungus genus	Stubble retrieved from	Sampling month									L[a]	Q	LSD[b]
		Jul	Aug	Sep	Oct	Nov	Dec	Jan	Feb	May			
Alternaria	Soil surface	2.5	2.5	0	1.25	0	5	0	0	3.75	NS	NS	4.90
	In soil	11.25	5	3.75	6.25	5	5	2.5	10	7.5	NS	NS	8.47
Aspergillus	Soil surface	0	1.25	0	7.5	0	0	0	0	0	NS	NS	4.89
	In soil	0	0	0	6.25	0	0	0	0	0	NS	S	2.32
Fusarium	Soil surface	5	15	13.75	7.5	7.5	32.5	18.75	3.75	5	NS	NS	12.02
	In soil	51.25	48.75	33.75	41.25	21.25	43.8	30	18.75	22.5	S	NS	21.54
Gliocladium	Soil surface	0	2.5	1.25	0	0	0	1.25	0	3.75	NS	NS	3.92
	In soil	1.25	1.25	6.25	7.5	6.25	7.5	2.5	11.25	10	S	NS	7.66
Penicillium	Soil surface	0	0	0	0	1.25	0	0	5	0	NS	NS	4.86
	In soil	0	2.5	1.25	3.75	1.25	0	0	0	0	NS	NS	3.72
Rhizoctonia	Soil surface	0	2.5	0	1.25	0	0	0	0	3.75	NS	NS	3.76
	In soil	10	11.25	18.75	20	13.75	3.75	10	16.25	16.25	NS	NS	10.18
Rhizopus	Soil surface	0	0	0	0	1.25	0	0	0	0	NS	NS	2.76
	In soil	1.25	7.5	1.25	10	6.25	7.5	2.5	7.5	2.5	NS	S	6.05
Stachybotrys	Soil surface	1.25	0	0	1.25	1.25	0	0	0	2.5	NS	NS	3.00
	In soil	13.75	26.25	27.5	31.25	32.5	38.8	18.75	11.25	25	NS	S	10.44
Trichoderma	Soil surface	2.5	7.5	11.25	7.5	2.5	0	1.25	0	1.25	S	NS	5.42
	In soil	0	21.25	11.25	12.5	16.25	15	6.25	17.5	8.75	NS	NS	11.40

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546 [a]L refers to a linear response and Q refer to quadratic response of the mean isolation frequency (%) for each fungus genus; NS non-significant; S significant ($P < 0.05$).

547 [b]Least significant difference ($P < 0.05$) of the mean isolation frequency (%) for each burial depth

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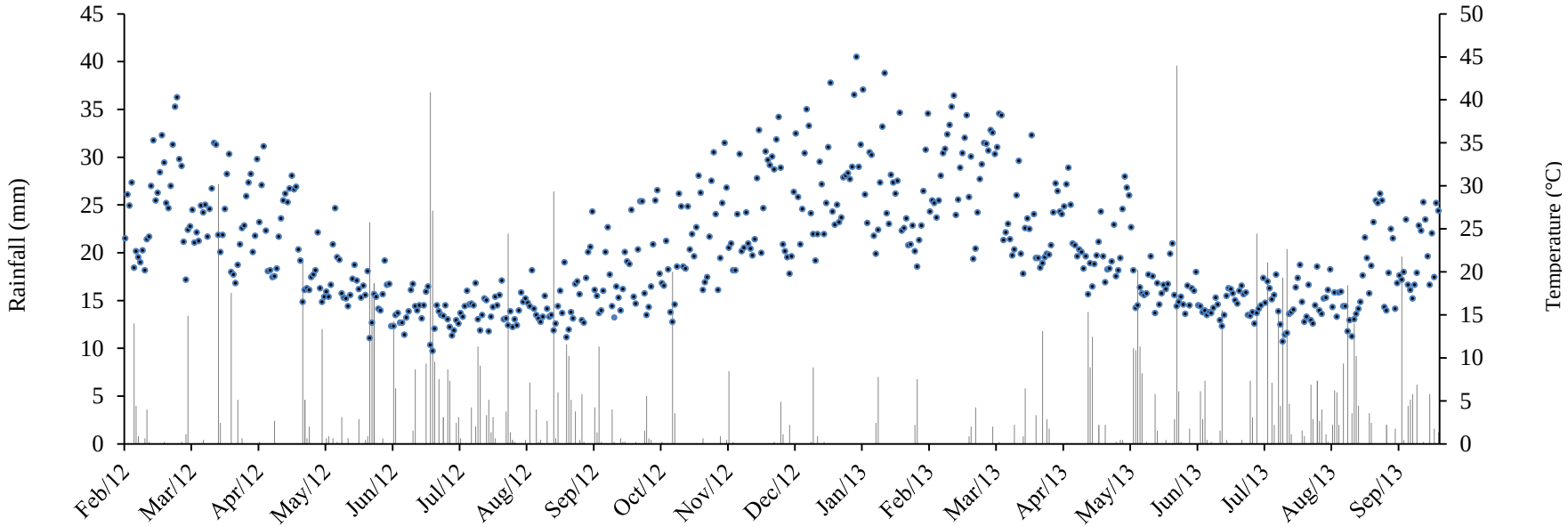
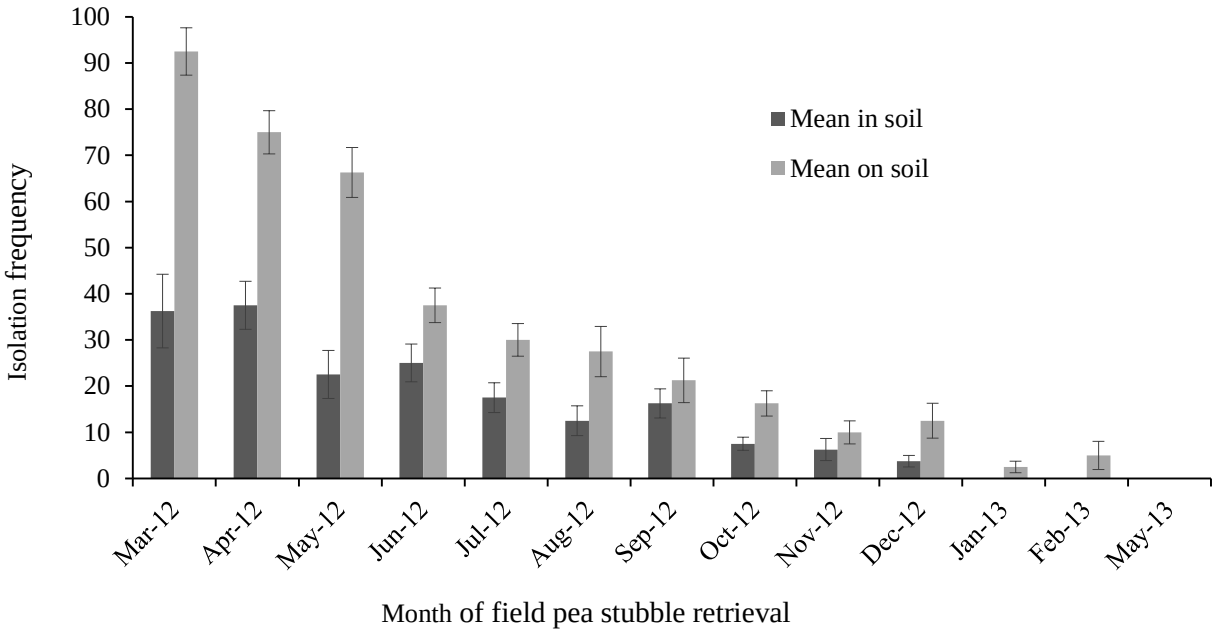
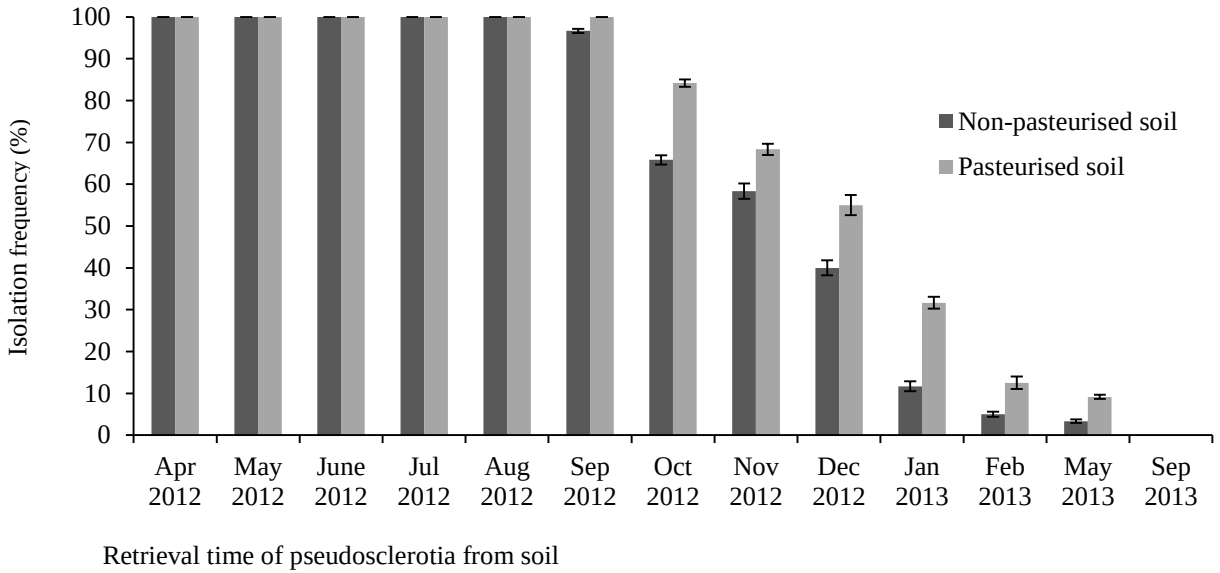


Figure 1 Mean daily air temperature (dark circle) and daily rainfall (grey column) at Waite Campus, South Australia for 20 months from February 2012 (data obtained from the Australian Bureau of Meteorology)



555

556 Figure 2 Mean isolation frequency of *Phoma koolunga* from infested field pea stubble buried in pots of field soil or left on
557 the soil surface in February 2012 over 15 months (mean of four stubble samples per treatment per month, bars represent
558 standard error)



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560 Figure 3 Percentage isolation frequency of *Phoma koolunga* from pseudosclerotial masses buried in field soil in pots in March 2012

561 over 18 months (bars represent standard error)

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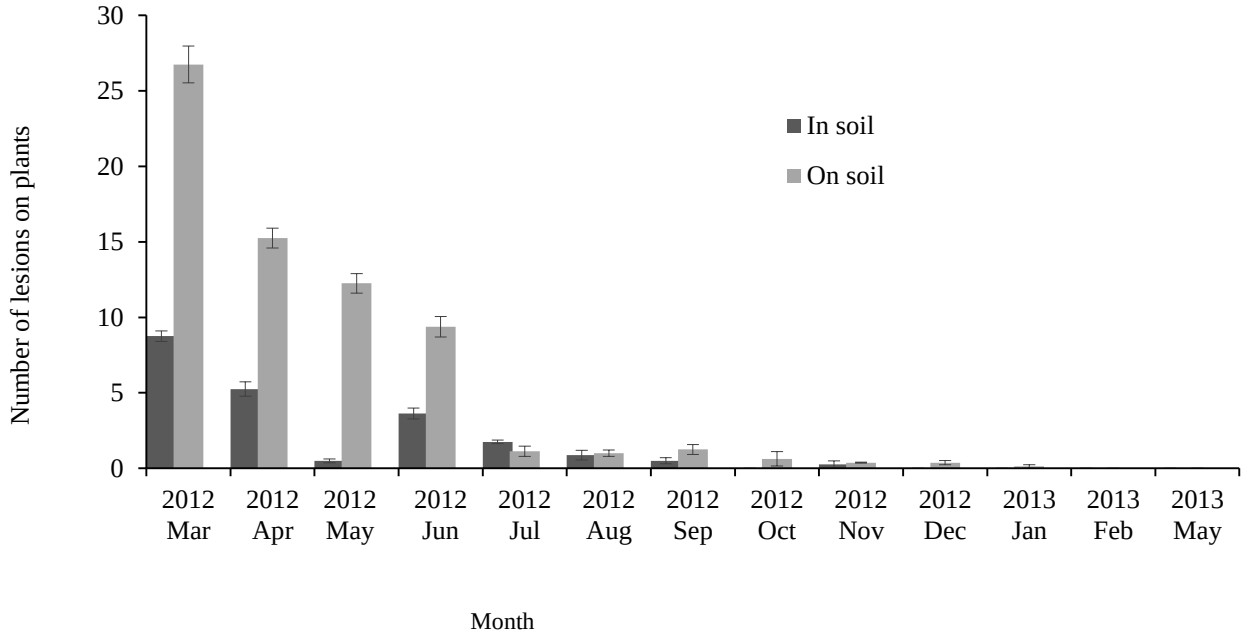
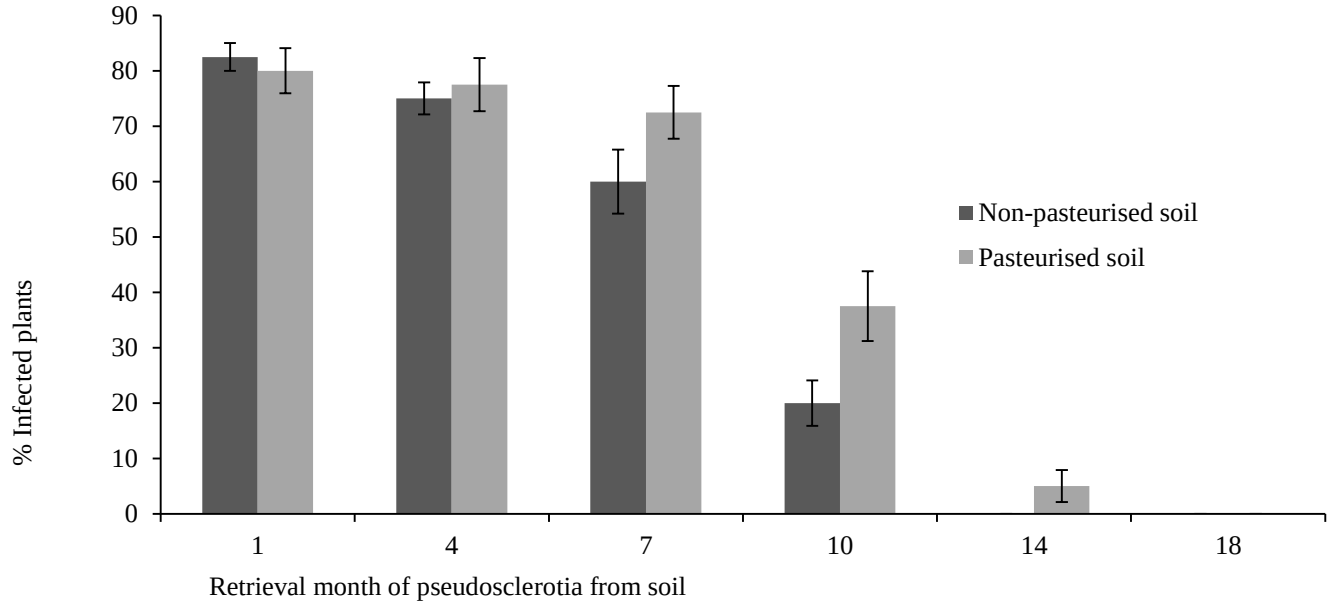


Figure 4 Mean number of ascochyta blight lesions on field pea plants sprinkled with milled stubble infested with *Phoma koolunga* retrieved from soil from one to 15 months after placement (bars represent standard error). There were no lesions on un-inoculated control plants.



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573 Figure 5 Percentage of field pea plants, in pots of soil, infected by pseudosclerotia retrieved from pasteurised or non- pasteurised soil

574 over 18 months (data are means of 40 plants, bars represent standard error)

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Chapter 3

Survival, transmission and control of *Phoma koolunga* in field pea seed and reaction of field pea genotypes to the pathogen

Manuscript prepared for submission to Australasian Plant Pathology

Survival, transmission and control of *Phoma koolunga* in field pea seed and reaction of field pea genotypes to the pathogen

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Abstract

Little is known about the epidemiology of *Phoma koolunga,* an important component of ascochyta blight of field pea in southern Australia. The aims of this research were to investigate seed infection, efficacy of fungicides as seed dressings and the reaction of current field pea genotypes to this fungus. The frequency of isolation of *P. koolunga* from seed samples from South Australia, Victoria and Western Australia ranged from 0 to 6 %. Disease was transmitted to 98 % of seedlings that emerged from artificially inoculated seeds (AIS) in growth room conditions. Seedling emergence rate from AIS was lower at 8°C than at 12, 16 and 20°C, and disease severity was greater at the lower temperature. P-Pickel T® and Jockey Stayer® were the most effective fungicides for reducing disease incidence and severity on seedlings emerged from AIS sown in soil and on germination paper, respectively. The response of 12 field pea genotypes to one isolate of *P. koolunga* was assessed by spraying plants with pycnidiospore suspension in controlled conditions and examining symptoms from 3 to 21 days post-inoculation (dpi). Genotypes Sturt, Morgan and Parafield showed more severe disease on the lowest three leaves than the other at 21 dpi. In another experiment, four genotypes of short, semi-leafless type field peas were inoculated with three isolates of *P. koolunga* which differed in virulence and assessed as described above. Kaspa showed significantly less disease than Morgan or WAPEA2211 at 21 dpi. Isolates of *P. koolunga* differed in aggressiveness based on % leaf area diseased until 14 dpi, but differences were not significant at 21 dpi.

Keywords Seed-borne, *Pisum sativum,* ascochyta blight, disease severity, seed treatment, resistance

Introduction

Ascochyta blight is one of the most important diseases of field pea (*Pisum sativum* L.) worldwide, including Australia, where it is estimated that crops suffer 15 % annual yield loss and up to 75 % in severe cases (Ali & Dennis, 1992; Bretag et al., 2006; Bretag & Ramsey, 2001; Davidson, 2012; Salam et al., 2011a). This disease is usually termed the ascochyta blight complex because it is caused by a number of closely related fungi, *Didymella pinodes* (*Mycosphaerella pinodes*), *Phoma medicaginis* var. *pinodella*, *Ascochyta pisi* and the recently characterised *Phoma koolunga* (Davidson et al., 2009).

D. pinodes, *P. medicaginis* var. *pinodella* and *A. pisi* are seed-borne (Bretag et al., 1995). Although seed infection by these pathogens rarely results in ascochyta blight epidemics (Moussart et al., 1998; Tivoli & Banniza, 2007), the use of infected seed usually leads to poor germination and death of seedlings due to foot rot (Wallen et al., 1967; Xue, 2000) or symptoms at or below the soil surface, especially at low temperature (Moussart et al., 1998). Nevertheless, the movement of infected seed not only spreads the fungi over long distance, but also has potential to disseminate compatible mating types of heterothallic fungi which could lead to the formation of teleomorphs in nature and increase the risk of development of new, virulent pathotypes (Kaiser, 1997; Moore, 1946; Tivoli & Banniza, 2007). Therefore, it is recommended that seed for sowing be accessed from areas with dry conditions or harvested as early as possible to avoid infection of seed (Hawthorne et al., 2012).

Although *Macrophomina phaseolina* had been reported to be seed-borne and to cause symptoms characteristic of ascochyta blight on field pea (Ali & Dennis, 1992; Ali et al., 1982), Davidson et al. (2009a) considered that the isolates tested by Ali and Dennis (1992) may have been mis-identified and, possibly, were *P. koolunga*. Therefore, it is possible that *P. koolunga* is seed-borne.

Seedling emergence rate and or yield are negatively correlated with the percentage of seeds infected (Gossen et al., 2010; Xue, 2000), therefore, treatment of seed with fungicides is an approach to reduce primary inoculum and seed to seedling transmission of ascochyta blight. Fungicides with the active ingredients thiram and thiabendazole, such as P-Pickel T®, Fairgro® and Reaper TT®, have been recommended to control ascochyta blight seed infection in pulse crops as well as seedling root rot for early sown fields or those in the vicinity of infested stubble (Hawthorne et al., 2010; Hawthorne et al., 2012; Kimber & Ramsey, 2001), but little is known about the efficacy of fungicide seed dressings in control of ascochyta blight caused by *P. koolunga.*

The development of ascochyta blight resistant field pea genotypes would be the most economical and long-term approach to control this disease but, despite considerable research, current genotypes are susceptible (Adhikari et al., 2014; Bretag et al., 2001; 2006; Davidson & Kimber, 2007; Khan et al., 2013; Tivoli et al., 2006a). Richardson et al. (2009) observed a significant interaction between 31 field pea genotypes and single isolates of ascochyta blight pathogens, including *P. koolunga*, for disease incidence and severity using a detached leaf method, but details were not reported. Evaluation of the response of current and new field pea genotypes to a range of isolates of *P. koolunga* is lacking.

The aims of this study were to investigate (i) aspects of seed infection in relation to *P. koolunga,* such as whether the fungus can be seed-borne and transmitted from seed to seedling, (ii) the most effective fungicides for seed treatment, (iii) the response of a number of current genotypes of field pea to inoculation with isolates of *P. koolunga.*

Materials and methods

Seed infection experiments

Confirmation of seed infection by P. koolunga

Field pea seed samples (cv. Kaspa) were collected in 2010 from the following sources; 18 National Variety Trials (NVT) in South Australia (SA), Victoria (Vic.) and Western Australia (WA), nine agronomy trials with three times of sowing in SA and four Pulse Breeding Australia (PBA) trial sites in SA. 'Kaspa' types, which are short and semi-leafless, make up more than 90 % of field pea crops in Australia (Hollaway, 2014). A modified agar plate method (Ali et al., 1982) to isolate seed-borne fungi was used to determine percentage of seed in each sample infected by *P. koolunga.* In brief, three replications of 100 seeds from each sample were surface sterilised in 2% chlorine solution for 2 minutes and were then dried on sterile filter paper. Ten seeds were placed in each plate contained potato dextrose agar (PDA) amended with 100 µg/l streptomycin. Plates were incubated at 22°C under fluorescent and near ultraviolet light; 12 h/12 h dark/light, for up to 21 days. The resulting colonies were identified *P. koolunga* based on morphological and microscopic cultural characteristics (Davidson et al., 2009) or DNA test (Ophel-Keller et al., 2008) when confirmation was required.

The same procedure was applied to examine infection of seed harvested from experimental potted field pea plants kept outdoors at Waite Campus, South Australia in November 2011. These plants had been artificially inoculated with mixed pycnidiospore suspensions (5×10^5 spores/ml) of four isolates of *P. koolunga*, 139/03, 142/03, 81/06 and FT07026, until run off at 3 weeks post-sowing. The inoculation process was repeated three times at 6-day intervals. These plants were severely diseased.

Examination of seed to seedling transmission of P. koolunga

Artificially inoculated seed (AIS) of field pea (cv. Kaspa) were prepared as described by Kimber et al. (2006). Briefly, surface sterilized seeds were submerged in mixed pycnidiospore suspension (1×10^7 spores/ml) of *P. koolunga* isolates 139/03, 142/03,

81/06 and FT07026, in a 2-L beaker within a 25-cm Kartell® implosion-proof desiccation bowl. The suction pressure was adjusted to -70 kpa and applied for 3 h. Seeds were then dried on sterile germination paper for 24 h in sterile conditions and stored at 4°C until use. The same procedure was applied to prepare control non-inoculated seed (NIS) except that water was used instead of spore suspension.

Seed to seeding transmission of *P. koolunga* was investigated using a method adapted from Kimber et al. (2006). Briefly, 40 AIS and ten NIS of cv. Kaspa were sown in University of California (UC) potting mix in Arborcell Easy-grower 50® trays with 50 cells per tray. The pots were immediately watered and maintained in a growth room under 12 h light/12 h dark at 16°C. After 2 weeks, when seedlings were about 3 cm high, the pots were covered with a plastic tent and an ultrasonic humidifier was applied for 4 days to maintain leaf wetness. Seedling emergence and disease incidence were assessed 2 weeks later when plants had approximately eight nodes. Foot rot and foliar disease severity were recorded using a 0 to 5 scale (Moussart et al., 1998) : 0, no symptoms; 1, streaks on the hypocotyl or on the epicotyl; 2, streaks on both the hypocotyl and the epicotyl; 3, lesions girdling the hypocotyl and streaks on the epicotyl; 4, lesions girdling both the hypocotyl and the epicotyl; and 5, weak plants with lesions girdling the hypocotyl and the epicotyl.

A necrotic index was calculated for each treatment by multiplying each disease score by the number of seeds in that category, and then dividing by the total number of germinated seeds (Moussart et al., 1998) as follows:

Necrotic index= $\sum_{i=0}^{n} n_i(d_i)/\mathrm{N}$

n_i refers to the number of seedlings in each disease category, d_i is the value of disease category and N is the total number of seedlings assessed.

The experiment was conducted as a completely randomised block design with four replications. This experiment was conducted twice. To study the development of disease on field pea plants infected via seeds, a similar experiment was conducted in a growth room in the conditions mentioned above except that the necrotic index was assessed 21 weeks after sowing, at physiological maturity, code 301 (Knott, 1987) when lower pods were dry.

Effect of soil temperature on seed to seedling transmission of P. koolunga

The seed to seedling transmission rate of *P. koolunga,* disease severity and lesion length on basal parts of field pea plants were studied at four soil temperatures (8, 12, 16 and 20°C) as described by Kimber et al. (2006). Each temperature was tested twice. Temperature affected the growth rate of the plants so plants in cooler temperatures were incubated for longer than those at warmer temperatures to enable all plants to reach the eight nodess growth stage for assessment.

Comparison of fungicides as seed treatment in soil or on germination paper

The efficacy of six fungicidal seed treatments, registered in Australia for pulse crops, was examined. The fungicides are listed here as product name (active ingredient) and dose per kg seed: P- Pickel T® (360 g/l thiram, 200 g/l thiabendazole) 2 ml, Thiram® (thiram 600 g/kg) 1.4 g, Jockey® Stayer™ (167 g/l fluquinconazole) 20 ml, Rovral® (500g/kg iprodione) 5 ml, Impact® (500 g/l flutriafol) 3 g and Sumisclex® 500 (500 g/l procymidione) 2 ml (Hawthorne et al., 2010; Sprague & Burgess, 2001). A method reported by Kimber and Ramsey (2001) using germination paper was applied to examine efficacy of these fungicides to control seed- borne *P. koolunga*. Twenty seeds of cv. Kaspa dressed with fungicide were placed on a 50 cm long and 20 cm wide sheet of germination paper in two rows 2 cm apart, the top row 5 cm below the upper edge of

157 the paper. The germination papers were rolled up and fastened by rubber bands, then
158 placed upright in plastic bags with 5 ml water at the bottom which were sealed and
159 incubated at room temperature (22°C) in the dark for 2 weeks (Kimber & Ramsey,
160 2001). The experiment was designed as a completely randomised block with four
161 replications and was conducted twice. Germination rate and disease severity on
162 seedlings were assessed as described above.

163 The six fungicides were also tested in a pot experiment conducted as completely
164 randomised blocks with six replicates in a growth room at 14-16°C. Every block
165 comprised eight pots (7 × 8.5 × 5.5 cm) containing UC potting mix, six of which were
166 sown with four AIS dressed with a fungicide each, one pot per fungicide. NIS or AIS
167 with no fungicide treatment were sown in the remaining pots as negative and positive
168 controls, respectively. The pots were randomized in each block and watered
169 immediately after sowing and thereafter when needed. Other conditions and disease
170 assessment at eight nodes growth stage were as described for the seed to seedling
171 transmission experiment. This experiment was conducted twice.

172 Reaction of field pea genotypes to *P. koolunga* isolates

173 The response of 12 field pea genotypes to one moderately virulent isolate of *P.*
174 *koolunga,* 139/03 (Davidson et al., 2009), was evaluated in an experiment conducted
175 using a design with four replicate blocks. Every block comprised three trays each with
176 12 pots. Each genotype was planted in a separate pot, four seeds per pot, within each
177 block. The pots were then placed in a growth room at 15 ± 1°C with 12h/12h light/dark
178 cycle. The plants were watered every 2 days. When all plants had at least four nodes,
179 they were sprayed until run-off with a spore suspension (5×10^5 spore/ml). Control
180 plants were sprayed with sterile distilled water. All plants were placed in a humidity tent

with a humidifier for 48 h after inoculation. Disease severity on the lowest three leaves of each plant was recorded at 2-day intervals starting at 3 days post-inoculation (dpi) to 11 dpi and then, due to fragility of plants, assessment was done at 5-day intervals concluding 21 dpi based on the 0-5 scale (Onfroy et al., 1999) and also as percentage of leaf area diseased (%LAD) (Priestley et al., 1985) at 16 and 21 dpi. Stem lesion length (mm) on internodes 2-4 was also recorded at 21 dpi. Area Under Disease Progress Curve (AUDPC) for each genotype was calculated using the formula of Shaner and Finney (1977) as follows:

$$\text{AUDPC} = \sum_{i=1}^{n} [(Y_{i+n1} + Y_i)/2][T_{i+1} - T_i]$$

Y_i = disease severity at the i*th* recording, Ti = days at the i*th* recording and n = total number of disease recordings. AUDPC of field pea genotypes inoculated with *P. koolunga* in this experiment was calculated at 21 dpi based on the 0-5 disease scale recorded from 3 to 21 dpi. To standardise, relative area under disease progress curve (RAUDPC) was calculated as AUDPC divided by the number of days from inoculation to recording of disease severity for the desired RAUDPC (Fry, 1978).

In the second experiment, four genotypes of short, semi-leafless type field peas that differed in susceptibility to *P. koolunga* isolate 139/03 were inoculated with three individual isolates of *P. koolunga* with low, medium and high virulence according to Davidson et al. (2009a). Methods were as above. The trial design was as for the first experiment of this series except with three blocks each comprising four trays. Each tray contained three pots of each genotype and the whole tray was inoculated with one isolate. Control trays were inoculated with sterile water. Disease severity was assessed as %LAD at 2-7 days intervals, ending at 21 dpi. Stem lesion length (mm) was assessed at 21 dpi. AUDPC and RAUDPC were calculated for each genotype/isolate combination.

206 **Statistical analysis**

207 Results for repeat experiments were combined, because t-test showed no significant
208 difference between repetitions. For the experiment of genotypes reaction to the fungus,
209 as the control plants were not diseased, these data were not included in analysis and
210 then it was analysed as randomized block. Also the results of other experiments
211 subjected to analysis of variance (ANOVA) using GenStat 15th edition SP2. Tukey's
212 honestly significant difference test at 95 % confidence intervals was applied to compare
213 means in each experiment.

214 **Results**

215 Confirmation of seed infection by *P. koolunga*

216 Most field pea seed samples collected in 2010 from pulse cropping areas in SA, Vic.
217 and WA were infected by ascochyta blight pathogens, mostly *Didymella pinodes*. *P.*
218 *koolunga* was isolated from most NVT samples from Vic. and SA but not from WA,
219 and from all PBA seed samples from SA (Table 1). Although the identification of *P.*
220 *koolunga* was based mainly on microscopic and morphological characteristics of
221 cultures, non-sporulating atypical colonies of *P. koolunga* were obtained and identified
222 by DNA testing. The frequency of isolation of *P. koolunga* from NVT seeds ranged
223 from 0 to 6.3 % and from PBA seeds from 0.3 to 5 %. *P. koolunga* was also isolated
224 from seeds harvested from agronomy trials with three times of sowing, most frequently
225 from plants sown early (30th April 2010) and least from plants sown late (11th June
226 2010). The seeds harvested from severely diseased plants that had been artificially
227 infected and grown at Waite Campus showed the most frequent infection, *P. koolunga*
228 being isolated from 36 % of seeds.

229

230 Examination of seed to seedling transmission of *P. koolunga*

231 Seeds artificially inoculated with conidial suspension of *P. koolunga* and maintained at
232 16°C showed a transmission rate to seedlings of about 98 %. The necrotic index
233 calculated for the seedlings with eight nodes was 1.89, as most seedlings had streaks on
234 both hypocotyl and epicotyl (Fig. 1). This increased to 3.32 at physiological maturity
235 due to development of lesions girdling the hypocotyl and the epicotyl on more than 53
236 % of plants. Streaks at physiological maturity ranged from 9 to 51 mm long, with mean
237 of 25.1 mm, and were dark and necrotic. A few infected seedlings were scored 5 as they
238 remained very short, about 7 cm, and weak with lesions girdling the epicotyl and
239 hypocotyl. Many pycnidia were observed on root systems or crowns, and sometimes 20
240 cm above the cotyledons, on 60 % of plants. Tall plants lodged onto soil or against
241 lower infected parts of plants, allowing the fungus to infect on aerial parts.

242 Effect of soil temperature on transmission

243 There was little effect of inoculation on germination except at 8°C, where it was
244 reduced by 7 %, as the rates of seedling emergence were 90, 91, 99 and 99 % and 97,
245 92, 100 and 100 % for AIS and NIS at 8, 12, 16 and 20°C, respectively. More than 29 %
246 of seedlings from AIS at 8°C had lesions which girdled the hypocotyl and or the
247 epicotyl and more than 3 % of seedlings were weak with lesions girdling both the
248 epicotyl and hypocotyl. These plants were usually stunted, 7-9 cm, compared to 23 cm
249 for healthy control plants. Lesions on roots and crowns were longer on seedlings at 8°C
250 than at other temperatures (Fig. 2), while no statistical difference was observed in lesion
251 length on seedlings at 12, 16 and 20 °C. Disease on seedling roots based on the 0-5
252 necrosis scale at 8°C was also significantly more severe than at other temperatures.

Many pycnidia were seen on most decayed seed coats of AIS in soil at 8°C at the time of recording disease severity while very few pycnidia were found at other temperatures.

Comparison of fungicides as seed treatment tested in soil or on germination paper

All fungicides treatments in potting soil reduced disease incidence and severity compared with the untreated AIS control (Fig. 3a). Disease incidence and severity on seedlings that emerged from untreated AIS (positive control) were 97 % and 1.68, respectively, whereas all seedlings from NIS (negative control) were healthy. There were no significant differences among fungicides in terms of disease incidence, whereas P-Pickel T® was more effective than Sumisclex® and Jockey Stayer® ($P < 0.05$) and as effective as the remaining three fungicides in reducing disease severity.

The efficacy of fungicidal seed treatments examined on germination paper differed from that in potting soil (Fig. 3b). Seedlings from AIS treated with Jockey Stayer® and rolled in germination paper were symptomless. The efficacy of P-Pickel T®, Rovral® and Thiram® was statistically similar to Jockey Stayer® based on disease incidence and severity. Impact® was less effective than the other fungicides, except Sumisclex®, for reducing disease incidence. All seedlings from NIS were symptomless and all from AIS without fungicide treatment were diseased. In addition to hypocotyl infection, seedlings germinated from AIS without fungicide treatment showed symptoms on 54 % of first internodes, 21 % of second internodes, 38 % of scale leaf and 8 % of first leaves.

Reaction of field pea genotypes to *P. koolunga* isolates

In the first experiment, the genotypes differed in reaction to *P. koolunga*, in that Sturt, Parafield, PBA Percy, Excell and Morgan were more severely diseased on leaves than the others and WAPEA2211, PBA Wharton, PBA Oura, Kaspa and PBA Twilight

showed the least disease at 5 and 7 dpi (data not shown). The same trend in reaction to the moderately virulent isolate 139/03 was seen at 21 dpi in terms of AUDPC and %LAD (Fig. 4), except that WAPEA2211 showed a greater increase in %LAD than the other genotypes and was one of the more susceptible genotypes at 21 dpi. Field pea genotypes could be grouped into several statistical categories ($P < 0.05$) on the basis of stem lesion length, such that Morgan had the most area infected of internodes, then Sturt and thereafter Excell, Parafield and Moonlight. Although WAPEA2211 had relatively short internode lesions, lesion length was not significantly different from those on PBA Wharton, PBA Twilight, Kaspa, PBA Oura, PBA Percy and PBA Gunyah.

In the second experiment there was an interaction between isolate and genotype (Fig. 5). Morgan was most severely diseased ($P < 0.05$) following inoculation with DAR78535 based on RAUDPC, but was statistically similar to WAPEA2211 for the other two isolates. Kaspa was least diseased ($P < 0.05$) for two isolates but similar to PBA Gunyah for isolate DAR78535. Despite this interaction, ranking of genotypes according to RAUDPC was the same or similar irrespective of the isolate. At 3 and 5 dpi %LAD on Morgan was greater than on Kaspa, but WAPEA2211 and PBA Gunyah were statistically similar to both Morgan and Kaspa. Thereafter, disease progressed more quickly on Morgan than on Kaspa; at 21 dpi 65 % of the leaf area of Morgan was covered with lesions and 42 % on Kaspa. The three isolates of *P. koolunga* differed in pathogenicity on leaves of the three genotypes early in the experiment, for example FT07026 was most and DAR78535 least aggressive in terms of %LAD at 3, 5, 10 and 14 dpi ($P < 0.05$), but they were statistically similar at 21 dpi. Stem lesions at 21 dpi were longer on Morgan inoculated with 139/03 ($P < 0.05$) than on the other three genotypes, and FT07026 and DAR78535 produced lesions on Morgan that were longer than those on Kaspa (Fig. 6).

Discussion

P. koolunga was widely distributed but infrequent in seeds collected in SA and Vic. Likewise, Davidson et al. (2009a) isolated this pathogen from aerial parts of field pea plants in many cropping regions of SA from 1995 to 2007 and Davidson et al. (2011) detected it in soil samples across SA and western Vic. The absence of *P. koolunga* from seeds from WA is in agreement with Tran et al. (2014), who did not identify *P. koolunga* among 1058 isolates of ascochyta blight pathogens from six locations in WA from 1984 to 1996, nor among 150 isolates from field pea leaves and stems in 2010, the year in which WA seed samples examined in our study were harvested. However, *P. koolunga* was isolated from up to 33 % of leaves affected by ascochyta blight-like symptoms in WA in 2012 (Tran et al., 2014). The fact that 78 % of SA seed samples yielded *P. koolunga* supports the report by Ali et al. (1982), in which 72 % of field pea seeds were reported to be infected by *M. phaseolina*, now thought to have been *P. koolunga* (Davidson et al., 2009). The decreasing incidence of infection of seed with later sowing in the agronomy trials in SA reflects disease severity reported by Davidson et al. (2013) which, in turn, was associated with rainfall and timing of ascospore release. More disease on plants is likely to lead to greater pod and seed infection, hence early sown crops are more likely to produce infected seed than later sown crops. The relationship between rainfall and seed infection should be examined in field conditions. As a teleomorph has not yet been recorded for *P. koolunga*, infected seed may act as source of primary inoculum to initiate disease in field pea cropping regions in Australia.

Plants arising from artificially inoculated seed developed lesions on the epicotyl and hypocotyl that eventually girdled the stem base, a phenomenon often referred to as foot rot, a common feature of the other ascochyta blight pathogens on field pea, particularly *P. medicaginis* var. *pinodella* (Knappe & Hoppe, 1995). Girdling increases the risk of

326 lodging and yield reduction. Gossen et al. (2010) reported that lodging was generally
327 more severe among plants that emerged from seed infected with *D. pinodes* when
328 incidence of seed infection was high. They also observed that seed infection with *D.*
329 *pinodes* can lead to reduced yield in some instances. Therefore reduced seedling
330 emergence due to *P. koolunga* at lower temperatures may impair establishment of plants
331 in the field and should be investigated.

332 The effects of temperature on transmission of *P. koolunga* from seed to seedlings
333 were generally in agreement with those presented by Moussart et al. (1998), in which
334 seedling emergence from AIS with *D. pinodes* at 8°C was lower than at 13 and 20°C.
335 The overall seed to seedling transmission rate at 8-20°C in this study was 98 %, much
336 more frequent than the 31% transmission rate from chickpea seed artificially inoculated
337 with *A. rabiei* at 5 to 19°C reported by Kimber et al. (2006). The necrotic indices for *D.*
338 *pinodes* reported by Moussart et al. (1998) on field pea seedlings at 8 and 12°C were
339 higher than at 20°C. Likewise, the most severe disease caused by *P. koolunga* on
340 epicotyl and hypocotyl of seedlings occurred at 8°C. Gossen and Morrall (1986)
341 reported that disease incidence on seedlings that emerged from infected lentil seeds was
342 greater in cold than warm soil, which was attributed to the seedling epicotyl growing
343 away from the infected cotyledons in warmer conditions. This may be the case also for
344 transmission of *P. koolunga* from seed to seedlings of field pea as the growth period to
345 eight nodes growth stage at 8°C was 10 weeks compared to 4 weeks at 20°C.
346 Furthermore, the observation that more pycnidia of *P. koolunga* developed on epicotyls
347 and hypocotyls at lower temperatures concurs with findings by Moussart et al. (1998)
348 for *D. pinodes*.

349 P-Pickel T® is the fungicide most commonly recommended for treatment of pea seed
350 to prevent seedling infection due to ascochyta blight in Australia as well as for control

of seedling root rot (Hawthorne et al., 2012). P-Pickel T® was generally the most effective of the six fungicides applied to treat seed infected with *P. koolunga* in soil. Thiram® has been shown to increase seedling emergence from seed infected with *D. pinodes* by 35-45 %, increasing yield compared with untreated seeds (Xue, 2000), and Agrosol® (thiram + thiabendazole, similar to P-Pickel T®) increased field pea yield more than Thiram® (Hwang et al., 1991). In the current study, seeds were submerged in spore suspension of the fungus for 3 h, resulting in 100 % infection, much greater than incidence of naturally infected seed. As a consequence, the fungicides may be more effective when applied to naturally infected seeds in the field than to AIS in pots. Although Jockey Stayer® controlled transmission of *P. koolunga* to seedlings on germination paper, producing 100 % healthy seedlings from AIS, it was less effective in soil, with 56 % disease incidence. This difference is not clearly understood.

This study showed that P-Pickel T® and Thiram® can be used as seed dressings for control of *P. koolunga* as well the other ascochyta blight pathogens without further costs to farmers. P-Pickel T® is most effective for crops with a high risk of ascochyta blight, for example where sown early, with a medium to high level of soil-borne inoculum, or close to last year's infested stubble as a source of wind-borne ascospores or pycnidiospores (Hawthorne et al., 2012; Salam et al., 2011b). Although the percentage of seed naturally infected with *P. koolunga* here was less than 10 %, seed harvested from fields with a history of disease, particularly in years conducive for development of disease, should be treated with a fungicide such as P-Pickel T®. Kimber et al. (2007) demonstrated that when a susceptible variety is sown, even 1 % of infected chickpea seedlings originating from seed infected with *D. rabiei* can act as foci for disease dissemination and lead to 60 % yield loss in conducive conditions. In contrast, Bretag et al. (1995), Moussart et al. (1998) and Gossen et al. (2010) found that seed

infection was not an important source of inoculum for epidemics, which Gossen et al. (2010) proposed was due to low frequency of transmission of *D. pinodes* from seed to seedling. Nevertheless, Gossen et al. (2010) demonstrated that it is crucial to avoid introducing *D. pinodes*, even at a very low level of seed infection, to regions in Canada that are free of ascochyta blight or that experience this disease infrequently. This would also apply to *P. koolunga* as the fungus is not prevalent in states of Australia other than SA and, recently, WA and seed to seedling transmission appears to be frequent. Although *P. koolunga* was not detected on field pea plants in WA before 2010, it was detected at several locations in 2012 (Tran et al., 2014), far from the SA border. One explanation for this rapid and broad distribution of *P. koolunga* to new areas in WA could be transmission by infected seeds, as seed harvested in SA, particularly for new genotypes, is sometimes transported to WA (Margetts, K., seednet.com.au, pers. comm., 2014). Alternatively, *P. koolunga* may have been present in WA for some years but not recognised. Therefore, the best measure to control long distance spread of this fungus is to use healthy seed obtained from dry regions for sowing in areas uninfected or with low infection. Furthermore, treatment of seeds with an appropriate fungicide such as P-Pickel T® or Thiram® can provide sufficient coverage against not only *P. koolunga*, but also other ascochyta blight pathogens and seedling root rot pathogens.

Most of the genotypes assessed in this study have been marketed to Australian farmers as moderately susceptible to ascochyta blight (Hawthorne et al., 2012), however there was some variation in susceptibility to *P. koolunga*. PBA Wharton, a newly released genotype, WAPEA2211, PBA Oura, Kaspa and PBA Twilight, when inoculated with a moderately virulent isolate of *P. koolunga*, exhibited less disease, based on %LAD, than the other seven genotypes tested. Davidson et al (2009) indicated that WAPEA2211 and Kaspa at 7 dpi were less susceptible than Parafield to *P. koolunga* in

401 controlled conditions, which has been confirmed here. Although WAPEA2211 has been
402 claimed as the first breeding line moderately resistant to ascochyta blight in WA and
403 was used as a benchmark in a study to compare susceptibility of new breeding lines to
404 *D. pinodes* (Adhikari et al., 2014), the lowest disease severity recorded by those
405 researchers on this line naturally infected in field conditions was 5.1 on a scale of 0-9,
406 while Kaspa was 6. This supports the results of our study, in that AUDPC and stem
407 lesion length on WAPEA2211 at 21 dpi were less than on Kaspa when inoculated with a
408 moderately virulent isolate of *P. koolunga*. The numeric value of %LAD at 21 dpi was
409 very similar to AUDPC for the 0-5 disease scale calculated over 3-21 dpi for each of the
410 genotypes assessed (Fig. 4), therefore it was concluded that %LAD was an accurate
411 assessment of disease severity. In addition this assessment was quicker than using the 0-
412 5 scale. Therefore, in the second experiment, disease severity was assessed using mean
413 %LAD over time to allow calculation of AUDPC and RAUDPC. In the second
414 experiment, the genotypes varied in response to isolates of *P. koolunga*. Results for
415 Morgan, the most susceptible genotype of the four tested in this experiment, were
416 similar to the first experiment. Davidson et al. (2009a) reported that severity of
417 ascochyta blight on WAPEA2211 inoculated with *M. pinodes* or *P. koolunga* was lower
418 than other lines tested at 7 dpi. In the current study, WAPEA2211 in both experiments
419 showed reduced susceptibility to *P. koolunga* soon after inoculation, but after 10 dpi this
420 effect disappeared. Leaves on WAPEA2211 seedlings expanded more slowly than other
421 genotypes while the fungus progressed at a similar rate on all genotypes, so the percent
422 necrotic area on WAPEA2211 leaves increased at a greater rate than on other genotypes.
423 A single assessment of length of stem lesion or girdling due to *D. pinodes* has been
424 suggested as sufficient to reflect differences in genotype susceptibility and as an
425 effective measure of disease development with time (Wroth & Khan, 1999). In this

study, Morgan had the longest stem lesions at 21 dpi, regardless of aggressiveness of the isolate, and the largest %LAD. Furthermore, Morgan was the tallest genotype in our second experiment; consequently, this genotype may be at most risk of girdling.

The pathogenicity of three isolates of *P. koolunga* on four genotypes differed in the first 14 days after inoculation, in accordance with results presented by Davidson et al. (2009a), but by 21 dpi the difference in aggressiveness had disappeared. These isolates developed at different rates on leaves, but as the rate of leaf area expansion was lower than disease progress, the differences in aggressiveness were not apparent after 14 dpi.

Tivoli et al. (2006b) considered assessment of partial resistance in growth room conditions to be more precise than in field conditions as the effect of environmental conditions is minimised. Given that, in this study, the response of field pea genotypes to inoculation with *P. koolunga* in two experiments was generally consistent and overall in agreement with Davidson et al. (2009a), it seems that screening in controlled conditions is a valid method to identify genotypes with reduced susceptibility to this pathogen. Further evaluation in the field conditions in Australia is necessary to assess the effect of environmental factors on response to *P. koolunga*.

The results of this research suggest that integrated strategies could improve management of ascochyta blight caused by *P. koolunga* on field pea plants. These strategies could include sowing of healthy seed, avoiding movement of infected seed to *P. koolunga*-free cropping areas, dressing seed with fungicides such as P-Pickel T® and, finally, choosing field pea genotypes with reduced susceptibility to this fungus. Efficacy evaluation of seed dressing in field conditions at different sowing times, using representative isolates of *P. koolunga* from SA, Vic. and WA on current and newly released field pea genotypes in different rainfall cropping areas, is warranted.

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Table 1 Percentage of field pea seed infected with *P. koolunga* harvested from trials or experiments at several locations in Australia in 2010 or 2011 (means of three replicates)

Seed source	State	Location	Mean	Standard deviation
NVT[a]	Victoria	Beulah	3.33	0.58
		Birchip	3.67	0.58
		Hopetoun	1.67	0.58
		Horsham	2.00	1.00
		Sea Lake	0.00	0.00
		Ultima	1.67	0.58
NVT	South Australia	Bool Lagoon	1.67	0.58
		Lameroo	0.00	0.00
		Laura	2.00	1.00
		Minlaton	3.67	0.58
		Mundulla	0.67	0.58
		Riverton	4.00	1.00
		Rudall	0.00	0.00
		Willamulka	1.67	1.53
		Yeelanna	6.33	1.53
NVT	Western Australia	Dalwallinu	0.00	0.00
		Pingrup	0.00	0.00
		Scadden	0.00	0.00
PBA	South Australia	Balaklava	5.00	2.65
		Kingsford	0.33	0.58
		Snowtown	2.33	0.58
		Willamulka	1.33	1.15
AT	South Australia	Hart,TOS[b] 30/04/2010	2.78	1.48
		Hart,TOS 21/05/2010	2.56	0.88
		Hart,TOS 11/06/2010	0.67	0.71
AIP[c]	South Australia	Waite Campus	35.67	5.51

[a] National Variety Trial (NVT), Pulse Breeding Australia (PBA), Agronomy Trials (AT)
[b] Time of sowing
[c] Artificially infected plants (AIP) in 2011

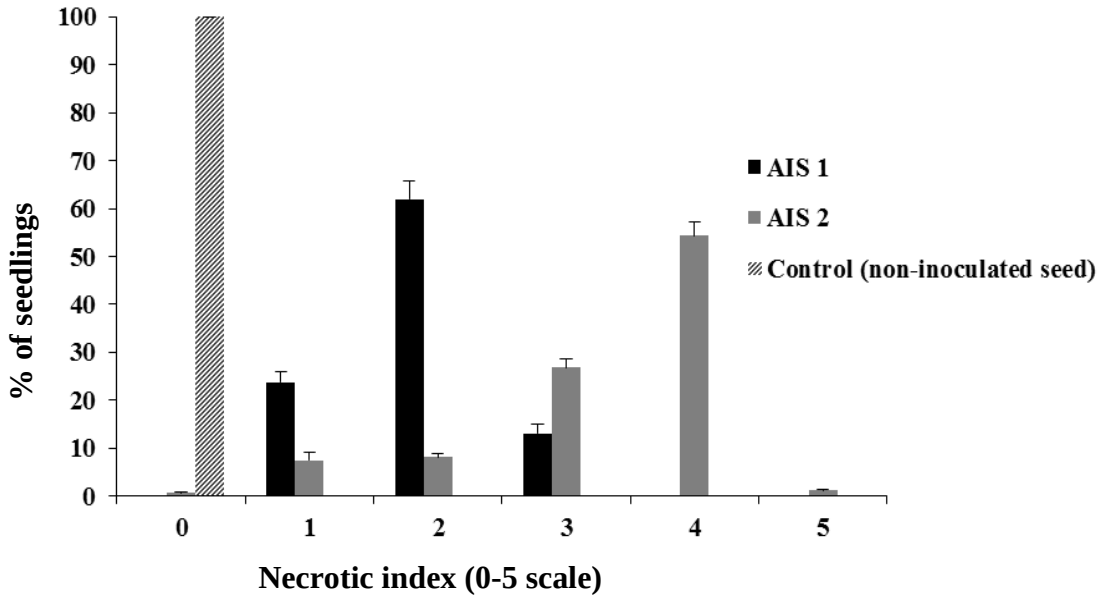

Fig. 1 Disease severity on field pea plants infected via seed artificially inoculated with *P. koolunga* and kept in a growth room at 16°C, assessed at eight nodes growth stage (AIS 1) and at physiological maturity (AIS 2), bars represent SE.

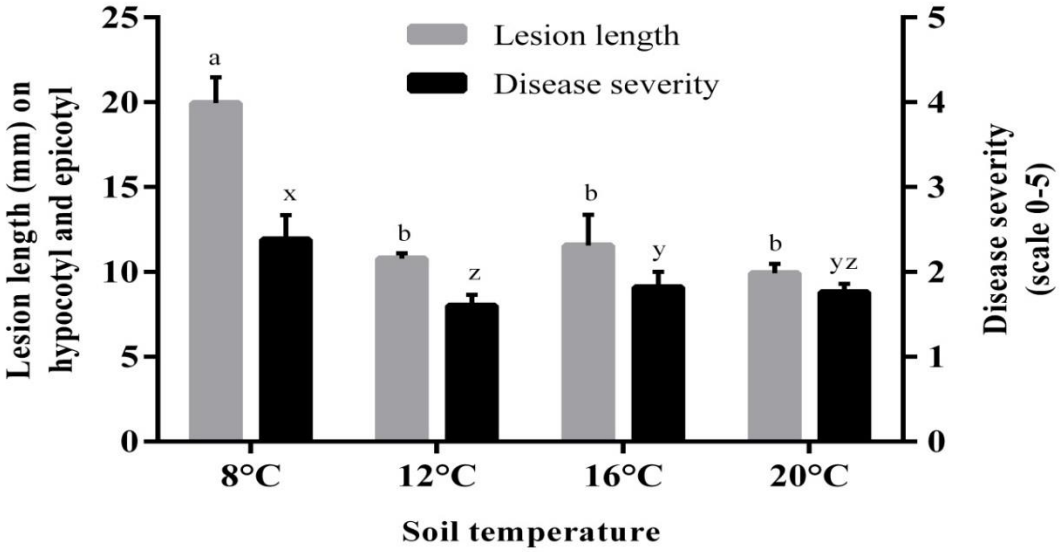

Fig. 2 Disease severity and lesion length on field pea seedling roots infected via seed artificially inoculated with *P. koolunga*, which were incubated in potting soil at four temperatures, transferred to a growth room and assessed at eight nodes growth stage. Bars with the same letters (a-b and x-z) are not significantly different by Tukey's test (P=0.05), bars represent SE.

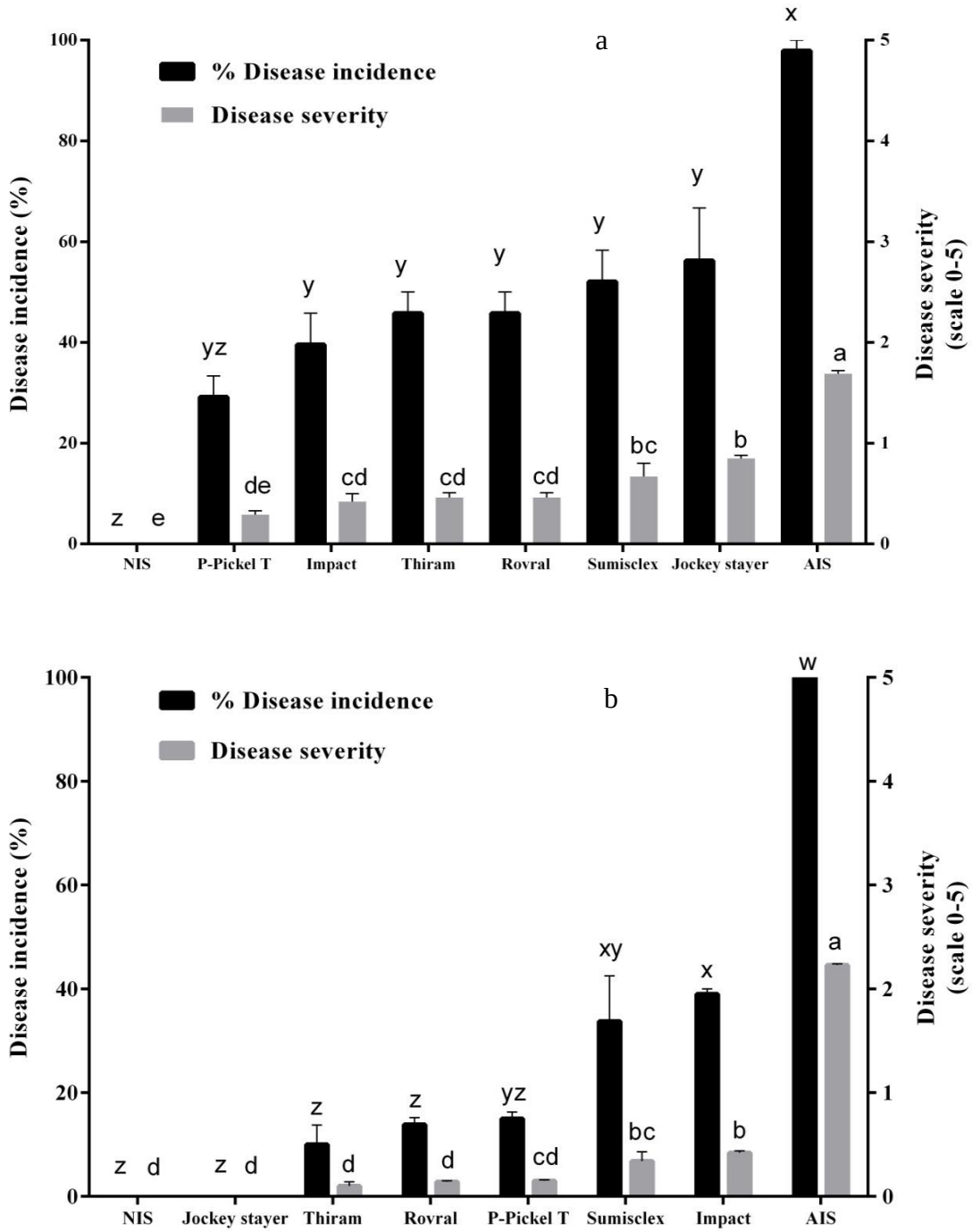

Fig. 3 Efficacy of fungicidal seed treatments on field pea seed artificially inoculated with *P. koolunga* and incubated in (a) potting soil and (b) on germination paper. Disease was assessed based on disease incidence (DI) and disease severity (DS) on seedlings roots. Treatment with the same letters (a-e and w-z) are not significantly different by Tukey's test (P=0.05), bars represent SE.

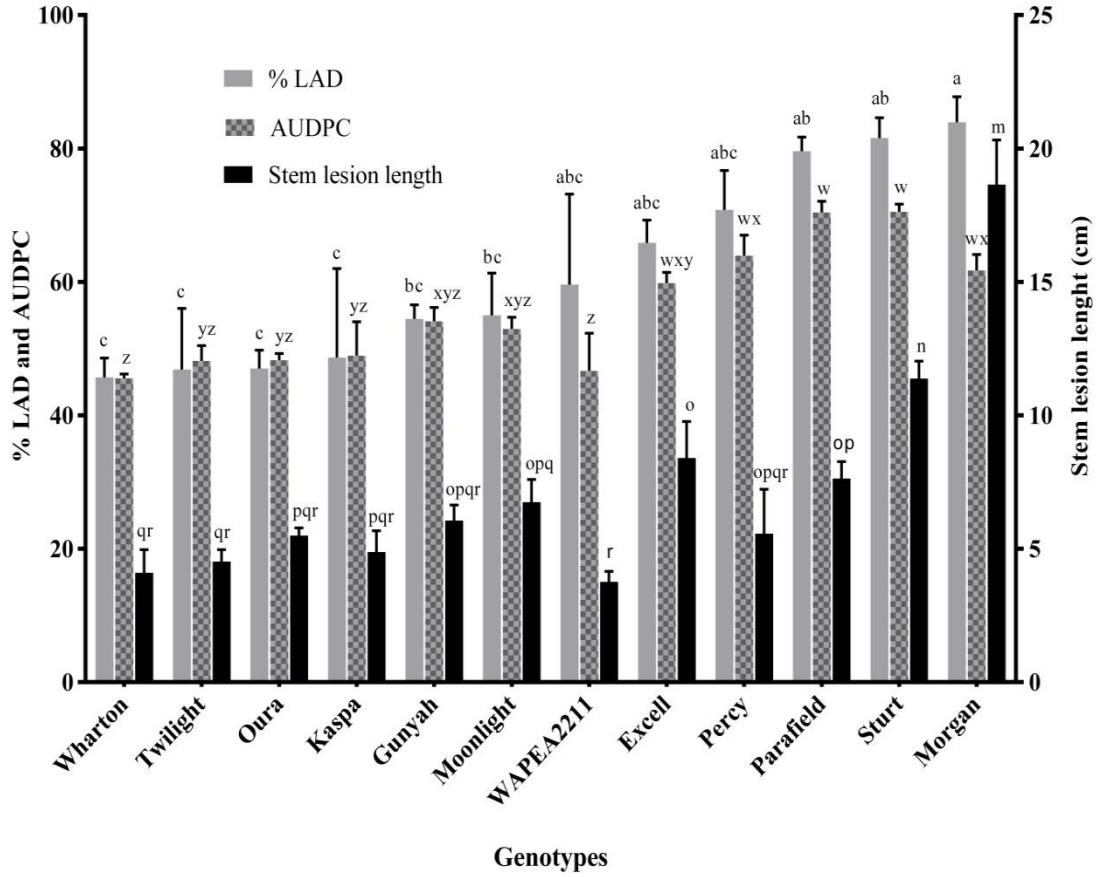

Fig. 4 Reaction of 12 field pea genotypes incubated in a growth room at 16°C after inoculation by spraying foliage with a spore suspension of *P. koolunga* isolate 139/03. Disease was assessed as % Leaf Area Diseased (%LAD), Area Under the Disease Progress Curve (AUDPC) and stem lesion length at 21 dpi. Means with the same letters (a-c, w-z, and m-r) are not significantly different by Tukey's test (P=0.05), bars represent SE.

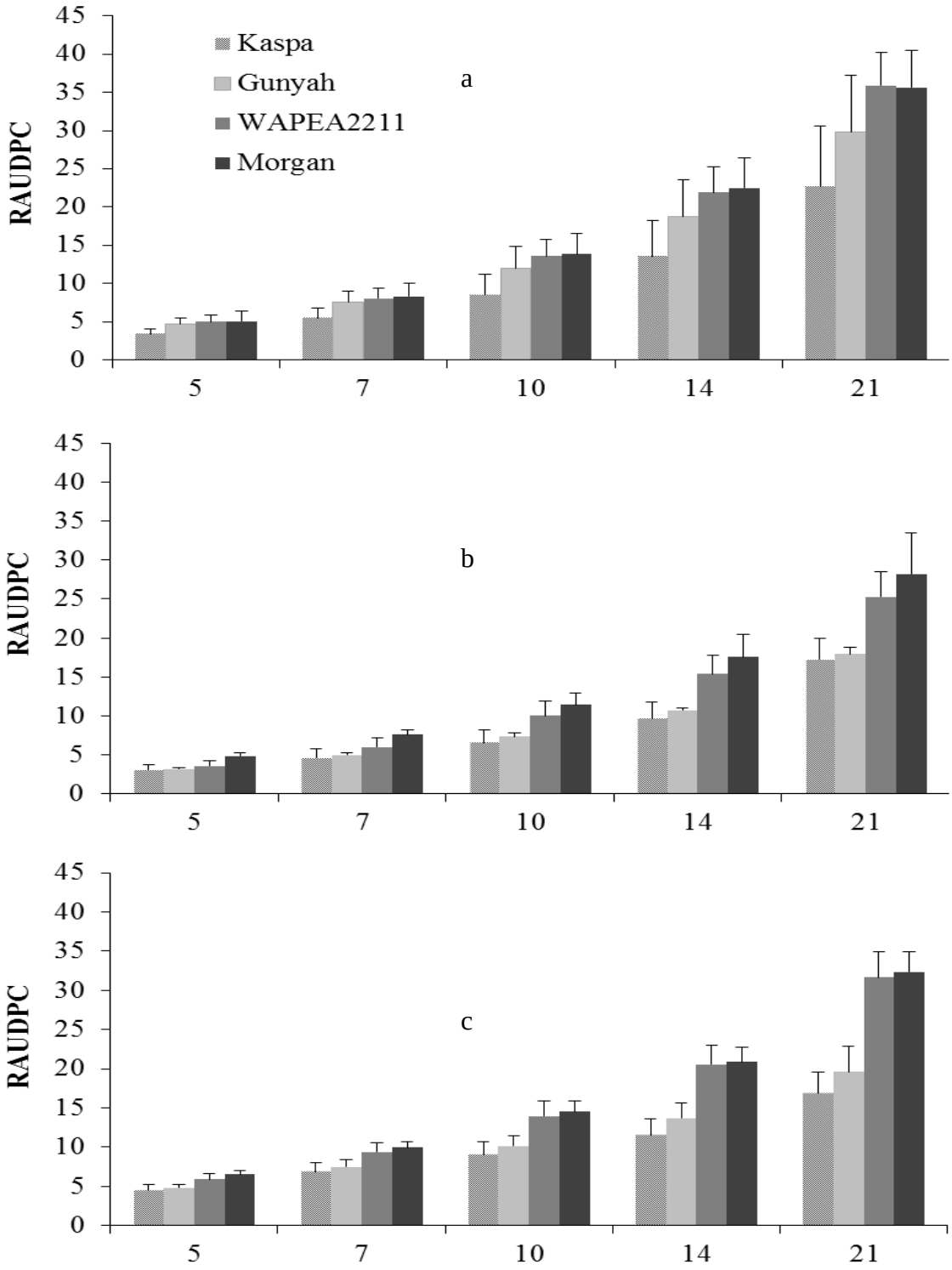

Fig. 5 Relative Area Under the Disease Progress Curve (RAUDPC) based on % Leaf Area Diseased on four field pea genotypes inoculated with spore suspensions of three *P. koolunga* isolates (a) 139/03, (b) DAR78535, (c) FT07026 and assessed to 21 dpi, bars represent SE.

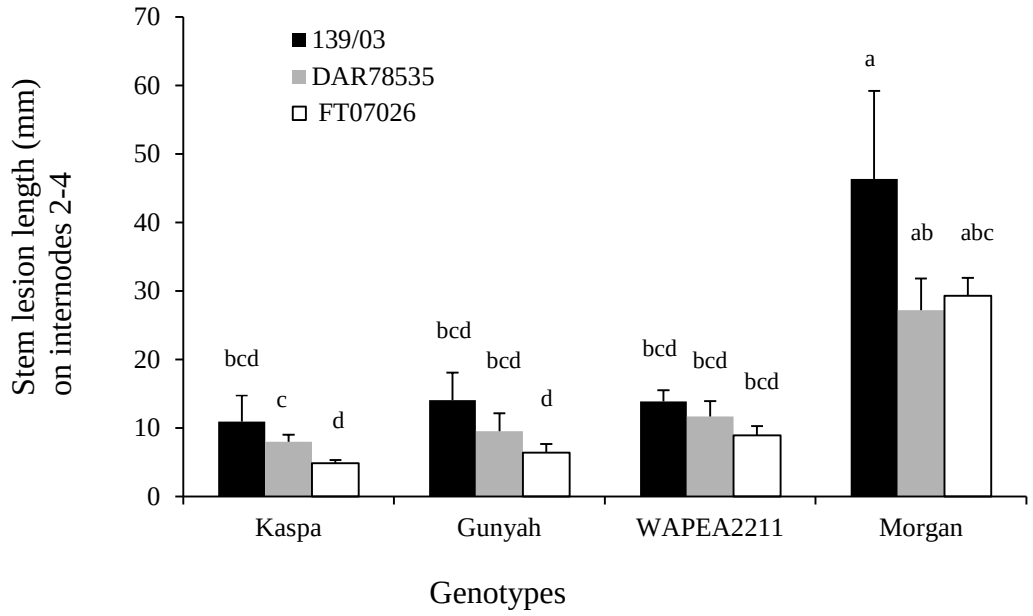

Fig. 6 Stem lesion length (mm) on four pea genotypes inoculated with spore suspensions of three individual *P. koolunga* isolates, 21 dpi in a growth room at 16°C. Means with the same letters are not significantly different by Tukey's test (*P*=0.05), bars represent SE.

Chapter 4

Appearance of atypical colonies of *Phoma koolunga* on culture media or artificially inoculated plants in controlled conditions

Manuscript prepared for submission to Mycologia

Appearance of atypical colonies of *Phoma koolunga* on culture media or artificially inoculated plants in controlled conditions

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Abstract

Phoma koolunga is a recently recognised causal agent in the ascochyta blight complex (blackspot) of field pea in Australia. Several isolates of this fungus exhibited atypical morphology and reproductive behaviour, including a rhizoid growth habit on media such as potato dextrose agar (PDA). Typical cultures of *P. koolunga* frequently formed rhizoid sectors on PDA. Rhizoid colonies were also isolated directly from field pea seeds plated on PDA to test for seed-borne infection. Atypical colonies grew more slowly than typical colonies on culture media. Dark pycnidium–like structures formed on mycelia of these atypical colony types as well as on inoculated plant material, and were similar to normal pycnidia of *P. koolunga* in terms of shape, size and color. However, each pycnidium–like structure contained thousands of round, hyaline guttulae of 0.4 -12.5 µm diam. Rhizoid colonies were purified by single hyphal tip isolation from sectors in cultures of *P. koolunga* or from atypical fungal growth from seeds on PDA plates amended with streptomycin. Purified rhizoid cultures were confirmed as *P. koolunga* by DNA test using *P. koolunga*-specific primers. Inoculation of the pure cultures onto field pea plants produced small lesions resembling ascochyta blight symptoms on leaves and stems.

***Key words*:** Ascochyta blight, *Pisum sativum*, pycnidiospore

INTRODUCTION

Ascochyta blight, one of the most devastating diseases of field pea (*Pisum sativum*), is widespread in most pea growing regions of the world, including Australia (Bretag & Ramsey, 2001). Yield loss due to ascochyta blight in Australia has been estimated at 15% annually (Bretag et al., 1995). This disease is termed the ascochyta blight complex as several fungi; have been reported as causal agents, and may occur together or

separately on one plant or even on one organ. *Didymella pinodes* (synonym: *Mycosphaerella pinodes*), *Ascochyta pisi* and *Phoma medicaginis* var. *pinodella* are prevalent components of this disease worldwide and *Phoma koolunga* is another pathogen in Australia characterised by Davidson et al. (2009a). Foliar symptoms caused by *P. koolunga* on field pea seedlings are identical to those produced by *D. pinodes*, however, develop more slowly.

Several isolates of *P. koolunga* from naturally infected field pea seeds exhibited atypical morphology and reproductive structures. The pycnidium-like structures were observed on stem sections cut from field peas and placed on filter paper which were artificially inoculated in vitro with typical *P. koolunga* isolates. These differences between typical and atypical colonies complicate diagnosis or identification of *P. koolunga* in routine experiments.

A sudden change in morphology and behavior of fungi has been defined as saltation or sectoring (Butler & Jones, 1949), a common phenomenon in fungi such as *Alternaria tenuis*, *Aspergillus terreus*, *Ceratobasidium cereale*, *Fusarium solani*, *Neocomospora vasinfecta*, *Phoma glomerata*, *P. herbarum*, *P. prunicola*, *P. radices* and *Rhizoctonia solani* (Mohendra, 1928, Rayner & Smith, 1929, Galloway, 1933, Dorenbosch, 1970, Abdalla, 1975a, Kataria, 1988). No single specific explanation has been given for this phenomenon to date. Hiroe (1937) described two types of sectoring in; the first type manifest, for example in *Brachysponum tomato*, as white mycelium that emerged spontaneously from black parental cultures but which was, otherwise, similar to the parent culture and did not revert. The second type has been reported in some species such as *Alternaria kikuchiana*, in which sectoring was affected by artificial treatments and sometimes reverted slowly or quickly to parental forms. Hiroe (1937) concluded that fungi change by saltation or mutation in the vegetative state. Some chemicals have

been shown to promote sectoring in some fungi, for example, *R. solani* and *F. solani* sectored on agar containing the fungicide feresan (20 % thiram) at 100 and 300 ppm, respectively (Abdalla, 1975a, Abdalla, 1975b) and *A. terreus* formed sectors on media amended with 30-50 ppm of salicylanilide (Galloway, 1933). Kataria (1988) reported that sectors of *C. cereale*, the wheat sharp eyespot pathogen, grew faster and had wider hyphae and higher favorable temperature than parent cultures. Those sectors also lost their ability to anastomose with the tester isolates but retained their pathogenicity on seedlings of wheat. This led Kataria (1988) to conclude that sectoring is a natural way for fungi to survive and adapt to high temperature, especially in the field.

As atypical colonies of *P. koolunga* appeared unpredictably, the aim of this research was is to describe how we isolated and identified atypical colonies of *P. koolunga* then compared their growth rate on synthetic media and virulence on field pea plants with that of parental cultures.

MATERIALS AND METHODS

Two atypical colonies were purified from single hyphal tips taken from sectored areas of otherwise normal cultures of *P. koolunga* (FT07026) and named here as FT07026-S1 and FT07026-S2. Three rhizoid-form colonies were also isolated from seeds embedded in PDA, coded here as YLS1, BLS1 and BES1. Colony diameter and morphological appearance of mycelia and pycnidium-like structures on 7-day-old cultures on PDA were recorded. All five atypical colonies were examined by DNA test using *P. koolunga*-specific primers designed by Davidson et al. (2009a) at the Root Disease Testing Service (RDTS) of the South Australian Research and Development Institute.

The structure and contents of pycnidium-like structures of FT07026-S1, YLS1 and BLS1 were examined by squashing them on slides and observing with a compound

microscope. To examine whether or not the contents of pycnidium-like structures were able to germinate, two such structures from each of three of the above-mentioned cultures were crushed in 1 ml sterile water and 100 µl of supernatant of the resulting suspensions from each culture were spread separately on a thin layer of water agar or PDA in Petri dishes. There were four replicate plates per suspension. The plates were kept at 22°C under 12 h fluorescent and near ultraviolet light and 12 h dark or exposed to natural light and examined for germination after 1, 2, 4 and 5 days using a dissecting microscope.

Mycelial growth of one typical isolate, FT07026, and FT07026-S1 which sectored from FT07026 was compared by transferring mycelial plugs from colony margins to four growth media. The experiment was designed as a completely randomised block with four replications. In every replicate, four 3-mm-diam. mycelial plugs of each culture were placed around the margin of each plate of oat meal agar (OA), PDA, malt extract agar and Sach's agar medium. Plates were sealed with parafilm and incubated under 12 h light/12 h dark and 22 °C for 7 days after which the diameter of the resulting colonies was measured.

To examine whether atypical colonies could revert to typical form, 3-mm-diam. mycelial plugs from10 day-old FT07026-S1 and YLS1 on PDA plates were subcultured to OA and incubated under 12 h light/12 h dark at 22° C. After 7 days morphological characters of mycelia and pycnidium-like structures were recorded.

Preliminary testing of atypical colonies for pathogenicity to field pea plants was conducted using a method adapted from Kimber (2011). Briefly, mycelial suspensions of typical isolate FT07026 and its atypical derivative, FT07026-S1, were sprayed onto two pots per colony, each pot containing five seedlings of field pea cv. Kaspa at the four nodes growth stage. Sterile water was applied to control plants. Plants were kept in a

humidity chamber for 4 days and observed for symptoms on leaves up to 14 day post-inoculation (dpi). As FT07026-S1 resulted in disease symptoms, an experiment was designed to compare pathogenicity of atypical and typical cultures. The mycelial plug method (Davidson et al., 2012) was used so that the amount of inoculum applied to field pea plants could be standardised. Despite numerous efforts, FT07026 could not be produced without pycnidia. Therefore, *P. koolunga* isolate 108/95, which is unable to produce pycnidia on PDA, was chosen as the typical culture for comparison. Eight mycelial plugs (3 mm diam.) from the margins of 7-day-old cultures of FT07026-S1 and from isolate 108/95 were placed on four leaves, one per leaf, of two seedlings, separately, at the four nodes growth stage. Un-inoculated control plants received sterile PDA plugs. Plants were kept in a humidity chamber for 4 days and the diameter of lesions on leaves was recorded up to 2 weeks post-inoculation (Davidson et al., 2012). A modified detached leaf method adapted from Onfroy et al. (2007) was also used for pathogenicity testing. Briefly, leaf detached from young seedlings of cv. Kaspa were surface-sterilised and placed adaxial surface uppermost on PDA plates. One 3-mm-diam. mycelial plug of FT07026-S1 or 108/95 was placed on the surface of each leaf and there were four plates, each containing two leaves, per fungus. Sterile PDA plugs were placed on un-inoculated control leaves. The experiment was designed as a completely randomised block with four replications. These plates were placed in plastic trays (31 × 44 × 6 cm), inside plastic bags (41 × 66 cm) which were sealed and incubated under 12 h light/12 h dark at 22° C for 7 days after which lesion diameter was measured. To confirm infection, re-isolation of the fungus from artificially inoculated tissues was attempted.

Results for experiments on colony growth on various culture media and pathogenicity tests of atypical and typical cultures were subjected to analysis of

variance (ANOVA) using GenStat 15th edition SP2. Tukey's honestly significant difference test at 95% confidence level was applied to compare means in each experiment.

RESULTS AND DISCUSSION

P. koolunga produced sectors in otherwise normal cultures on PDA (FIG. 1A) which were rhizoid in habit and different from the radial growth of typical *P. koolunga* (FIG. 1B). Rhizoid colonies were obtained upon subculture of normal isolates from various sources and directly from seed embedded in PDA during testing for infection by *P. koolunga*. Rhizoid colonies were also observed on stem pieces excised from field pea and artificially inoculated in vitro with spore suspensions of *P. koolunga* and placed on PDA, dilute V8 Agar or sterile filter paper. Sectoring from typical cultures of *P. koolunga* growing on PDA plates occurred frequently (FIG. 1A). The atypical cultures did not revert to typical form when subcultured on OA. All five purified atypical cultures analysed by DNA test in the RDTS laboratory were confirmed to be *P. koolunga*.

Dark pycnidium-like structures were formed on mycelia of atypical colonies (FIG. 1B) as well as plant material inoculated with atypical cultures (FIG. 2) and were similar to typical pycnidia of *P. koolunga* isolate FT07026 in terms of shape, color and size, but did not contain pycnidiospores. Atypical sectors did not form on OA when used as growth medium for typical colonies and only fertile pycnidia with pycnidiospores formed on this medium. This is in agreement with Dorenbosch (1970), who reported that OA stimulates the formation of pycnidia and pycnidiospores of *Phoma*-like fungi. Davidson et al. (2009a) described typical 7-day-old colonies of *P. koolunga* on malt agar to be 12 mm diam., gray to white color, irregular with compact fine cottony white

to pale gray aerial mycelium, but occasionally olivaceous with little or no aerial mycelium. Typical pycnidia on OA are 150-210 µm diam., globose usually with an elongated neck, scattered on or immersed in agar and pycnidiospores of typical colonies are hyaline, ellipsoid to oblong, 12.5-19.5 × 5-7 µm diam., often unicellular, occasionally with one septum (Davidson et al., 2009). In comparison, pycnidium-like structures formed on atypical colonies contained thousands of round, clear structures of various sizes (FIG. 3), usually smaller (0.4 -12.5 µm diam.) than normal pycnidiospores of *P. koolunga*. These structures were fatty guttulae, which occur in other *Phoma*-like species in pycnidia but also in pseudothecia, and remain sterile (de Gruyter, 2013, pers. comm.). Mohendra (1928) also reported the formation of sterile pycnidia by saltants of *Phoma* species. Butler and Jones (1949) reported that sectors may differ from the parental form in characters such as spore production, chlamydospore and sclerotium formation as well as growth rate. The saltants or sectors in Mohendra's research did not revert to the parent form when re-cultured on PDA or OA and failure to revert to typical form was also reported for sectors of other fungi such as *Brachysponum tomato*, causal agent of leaf blight of tomato (Hiroe, 1937), *C. cereale* (Kataria, 1988), *F. solani* (Abdalla, 1975a) and *P. radices* (Rayner & Smith, 1929). Likewise, atypical cultures of *P. koolunga* failed to revert to the typical form when sub-cultured on OA.

Typical colonies of *P. koolunga* grew faster ($P < 0.05$) than atypical colonies on growth media; the mean diameter of typical colonies on OA, PDA, malt extract agar and Sach's agar after 7 days was 24.5 ± 0.22, 28.5 ± 0.18, 18.3 ± 0.22, 15.8 ± 0.23 mm, respectively, in comparison to 18.5 ± 0.22, 21.3 ± 0.18, 14.8 ± 0.23, 10.3 ± 0.39 mm for atypical colonies.

Atypical cultures of *P. koolunga* were able to infect field pea plants; however, the lesions were smaller than those caused by typical cultures when tested by all three

methods. For example, the size of lesions caused by mycelial plugs from atypical and typical colonies on detached leaves at 7 dpi *in vitro* was significantly ($P < 0.05$) different, 5.9 ± 0.17 and 9.8 ± 0.1 mm, respectively. FT07026-S1 was less virulent than 108/95m which may suggest that atypical cultures of *P. koolunga* are less virulent than typical cultures. A reduction of virulence of cultures derived from sectors has also been reported for other fungi, such as *F. solani* on *Petunia* (Abdalla, 1975a). In our study, pycnidium-like structures formed profusely on old leaves and stems of field pea plants which had been artificially inoculated with mycelial suspensions of atypical colonies and maintained at 14-16° C in a growth room for 3 months. These pycnidium-like structures contained fatty guttulae, not pycnidiospores.

Sectoring or saltation seems to be a feature of *P. koolunga,* as it is for many other fungi. Morphology differed from parent cultures, but sectors retained pathogenicity to field pea plants although virulence was compromised. Although no reason was found for sectoring and formation of sterile pycnidium-like structures on culture media or living field pea plants, it is important to know that this fungus can appear as atypical colonies in culture as this can make it difficult to identify *P. koolunga* in experiments. It would be of interest to know what causes atypical growth of *P. koolunga*, especially in primary isolates directly from plant material.

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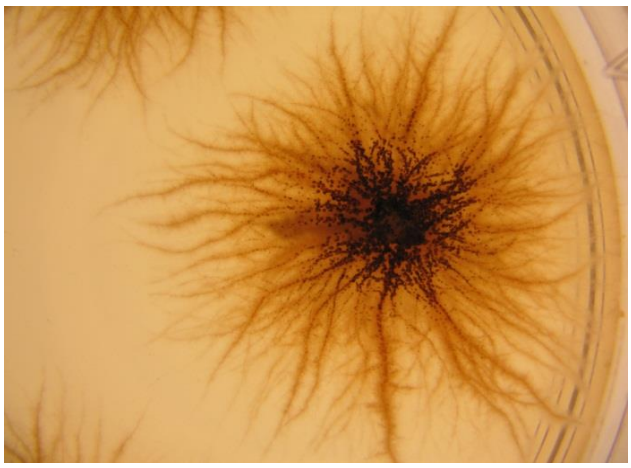
A



B

FIG. 1. Sectoring of atypical rhizoid mycelium (arrow) from typical colony of *Phoma koolunga* isolate FT07026 on PDA (A) and pycnidia-like structures on rhizoid mycelia of atypical colony, FT07026-S1 on PDA (B)

A

B

FIG. 2. Pycnidia-like structures formed on field pea stem artificially inoculated with a mycelial suspension of atypical colony of *P. koolunga* sectored from isolate FT07026

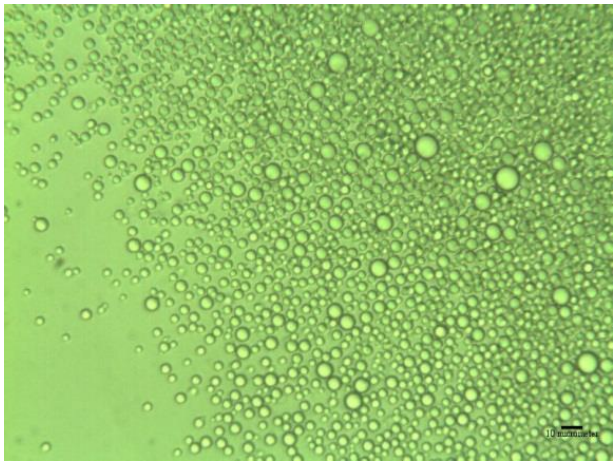


FIG. 3. Fatty guttulae released from pycnidium-like structure from atypical colony of *P. koolunga* FT07026-S1 formed on PDA (scale bar = 10 µm)

Chapter 5

Investigation of formation of teleomorph of *Phoma koolunga* in controlled conditions

5.1 Introduction

Ascochyta blight is a complex disease on field pea with worldwide distribution caused by four closely related pathogens; *D. pinodes*, *Phoma medicaginis* var. *pinodella*, *Ascochyta pisi* and the recently identified *P. koolunga*. These fungal pathogens survive on pea stubble, soil and seed. The teleomorphs of the first three species have been reported. *Didymella pinodes* is the sexual stage of *Ascochyta pinodes*, which forms pseudothecia on stems and pods of field pea (Punithalingam & Holliday, 1972a, Peever et al., 2007). Bowen et al. (1997) reported that the teleomorph of *Phoma medicaginis* var. *pinodella* was formed in laboratory conditions, however attempts by other researchers to reproduce the teleomorph of this fungus were unsuccessful (Onfroy et al., 1999, Tivoli and Banniza, 2007). *Didymella pisi* was described as the teleomorph of *A. pisi* by Chilvers et al. (2009), after they were able to initiate formation of pseudothecia on pea stubble in controlled conditions. Despite those reports, neither pseudothecia nor ascospores of these latter species have been reported on pea plants in field conditions (Bowen et al., 1997, Chilvers et al., 2009). *A. pisi* is heterothallic and Bowen et al. (1997) suggested that *P. medicaginis* var. *pinodella* is also heterothallic as they were unable to produce pseudothecia from single ascospores. *D. pinodes* is homothallic (Punithalingam & Holliday, 1972b).

It has been well documented by several researchers that ascospores of *D. pinodes* released from ascocarps formed on pea stubble play an important role in initiation and severity of disease in field pea crops in the vicinity of the previous year crop debris (Davidson & Ramsey, 2000, Bretag & Ramsey, 2001, Galloway & McLeod, 2001, Bretag et al., 2006, Salam et al., 2011, Davidson et al., 2013). These ascocarps not only produce ascospores as primary inoculum to initiate

disease, more significantly, they also deliver new combinations of genes and therefore possible new virulent pathotypes on hosts or genotypes resistant to frequently applied fungicides (Bowen et al., 1997). As a consequence, in the presence of the teleomorph of a pathogenic fungus, disease control may require modification according to the time and number of ascospores released in a cropping season.

Chilvers et al. (2009) reported a successful method for production of the teleomorph of *D. pisi* on field pea stubble. They submerged pea stem pieces in conidial suspension of a mixture of two isolates of compatible mating types for one hour and then placed the stem pieces on wet filter paper in 9 cm Petri plates at room temperature for 3 days before incubating them at dark for 8 weeks at 10 °C. The same method was chosen by Trapero-Casas and Kaiser (1992) after several negative attempts to initiate formation of ascocarps of *D. rabiei* on artificial media. Bowen et al. (1997) used corn leaf segments instead of field pea stems to promote production of pseudothecia of *P. medicaginis* var. *pinodella*. In comparison, various growth media and incubation conditions, including light or darkness and temperature, have been studied by several researcher in order to find an efficient method to produce the teleomorph of a range of fungal species (Barnett, 1953, Krause & Webster, 1972, Tsuda & Ueyama, 1981, Klittich & Leslie, 1988, Mengistu et al., 1993, Mengistu et al., 1995, Rubiales & Trapero-Casas, 2002, Friesen et al., 2003, Kent et al., 2008, Choi et al., 2009, Morita et al., 2012). For example, Choi et al. (2009) found that 10% V8 juice agar and carrot agar, 23°C and 12 h near ultra violet (NUV) and fluorescent light/12 h dark were the optimum condition for sexual reproduction of *Fusarium fujikuroi*; different combinations of concentrations of V8 or water agar and synthetic nutrients with

low agar, temperature range from 18-28 °C and light conditions were less effective to initiate production of large number of perithecia on plates.

P. koolunga is heterothallic (Lichtenzveig 2013, pers. comm.) like most of the other ascochyta blight pathogens of legumes, including *A. rabiei*, *A. pisi*, *A. fabae*, *A. lentis*, *A. vicia villosae* and *A. pinodella* (Punithalingam & Holliday, 1972a, b, Punithalingam & Gibson, 1976, Punithalingam, 1980, Jellis & Punithalingam, 1991, Trapero-Casas & Kaiser, 1992, Bowen et al., 1997, Chilvers et al., 2009), therefore, it needs two compatible mating types for fertilisation and production of sexual fruiting bodies.

So far, the teleomorph of *P. koolunga* on plant materials or on culture medium has not been reported. Therefore, this study investigated the possibility of formation of the teleomorph of this fungus in laboratory conditions on pea stubble and in culture on different artificial media.

5.2 Materials and Methods

5.2. 1 Experiments with multiple *P. koolunga* isolates

The method described by Trapero-Casas and Kaiser (1992), with some modifications, was used to examine the formation of pseudothecia of *P. koolunga*. Nineteen isolates of *P. koolunga* were inoculated on PDA plates and incubated under 12 h black and fluorescent light / 12 h darkness cycle. After 2 weeks, 5 ml of sterile water was added to each plate and then pycnidiospores were harvested by scraping the surface of the agar. The concentration of spores in suspensions of each individual isolates was adjusted to approximately 5×10^5 spores ml^{-1}. Healthy stems of field pea plants harvested from the glasshouse were cut into 6-cm long pieces and were autoclaved twice at 121° C for 20 min. A 5 ml spore

suspension of a mixture of two different isolates was placed in a 10 ml sterile tube which was labelled accordingly. All possible pair-wise combinations of the 19 isolates of *P. koolunga* (171 tubes) were treated in the same manner. A 5 ml spore suspension of each of the individual isolates was placed separately into extra 19 sterile tubes. Four pieces of autoclaved field pea stem were placed in each 10 ml tube of spore suspension of paired or single isolates for 1 hour. Stem pieces were then drained and placed on moist sterile filter paper in 9-cm Petri plates which were labelled accordingly. These plates were placed in white clean trays (31 × 44 × 6 cm) and inside plastic bags (41 × 66 cm) with the opening sealed by folding. The trays were incubated in darkness at 20°C for 5 days and then at 8°C up to 8 weeks. Every 2 weeks, 4 ml sterile distilled water was added to each plate to maintain high humidity. After 8 weeks, all stem pieces were examined using a dissecting microscope for structures that may be fruiting bodies. Slides of structures that resembled pseudothecia or pycnidia were prepared and examined using a compound microscope. As the results of this experiment were negative, the experiment was repeated using the same procedure except that plates were incubated under a cycle of 12 h UV and fluorescent light and 12 h darkness at 8 °C to examine whether black and white light may have a role in formation of pseudothecia, as the presence of black light with white light is necessary for production of pycnidia of this fungus (Davidson et al., 2009a).

5.2.2 Experiments with *P. koolunga* isolates representing two mating types

As attempts to initiate formation of pseudothecia of *P. koolunga* by the methods described above were unsuccessful the following media and incubation conditions were tried. Isolate DAR78535 was used as a representative of mating type 1 and isolate FT07013 as mating type 2 (Lichtenzveig 2013, pers. comm.) in the

following experiments. To provide a wide range of nutrient and environmental factors for sexual reproduction of *P. koolunga*, various growth media, temperatures and light conditions reported for closely related ascomycetes were examined in this study. For each light/temperature treatment, three plates were used and for dark conditions, plates were covered by aluminium foil (Friesen et al., 2003). The conditions included continuous darkness; 12 h UV and fluorescent light/12 darkness, 12 h UV light/12 h darkness (Choi et al., 2009) at four temperatures, 8, 15, 22 and 29 °C. In total, 48 combinations of growth medium, light and temperature were tested for crossing of the two isolates representing the two mating types.

Using a method modified from Krause and Webster (1972), two double autoclaved 6-cm long pieces of field pea stem were put on 10% V8 agar with pH 6 and 7.5, and a 5-mm diameter plug of each mating type grown on PDA was placed at each end of each stem piece.

Also, based on the method of Mengistu et al. (1995) for production of ascocarps of *Leptosphaeria maculans*, a 5-mm diameter plug of each of two isolates of *P. koolunga* of different mating types were placed 5 cm apart on 10% V8 agar and incubated at 22 °C with a 12 h photoperiod. After a week, 10 ml of 1.5 % water agar was poured on to cover the cultures and plates were sealed and incubated as mentioned above under different light and temperature conditions.

Carrot agar was used to initiate production of pseudothecia of *P. koolunga* using methods described by Barnett (1953) and (Choi et al., 2009). Mycelial plugs of each mating type of *P. koolunga* were point-inoculated at three positions on carrot agar in each 9-cm plate and incubated at 22 °C. After a week, spore suspension (1×10^5 conidia/ml) of the other mating type was added to the plate

and incubated for 8-12 weeks in the light and temperature conditions mentioned above.

5.3 Results

Many black spherical structures were formed on all the field pea stem pieces on filter paper or growth media. It was not easy to differentiate pycnidia from structures that may have been pseudothecia using a dissecting microscope, therefore, representative structures were examined using a compound microscope. Although many pycnidia were formed on stem pieces or on the media described above (section 5.2), no pseudothecium was found on any of those plates.

5.4 Discussion

As all attempts to initiate formation of pseudothecia of *P. koolunga* were negative, conditions conducive for formation of pseudothecia have yet to be identified. However, all possible paired combinations of 19 isolates of this fungus examined failed to produce pseudothecia. One likely explanation for this failure is that none of those 19 isolates was "female fertile". Formation of sexual structures of heterothallic species requires not only two mating types but, most importantly, one mating type needs to be able to act as the female fertile parent for production of ascocarps (Leslie & Summerell, 2006, Walsh et al., 2010). Although the two isolates, DAR78535 and FT07013, used belong to different mating types, it is possible that neither of them is female fertile. This explanation may be extended to all other isolates which belong to mating type 1 or 2. *P. koolunga* is a relatively recently described species, which has been reported mainly from South Australia (Davidson et al., 2009a) and infrequently from Victoria and Western Australia (Davidson et al., 2011). The limited distribution, recent characterisation and probably, recent emergence of this pathogen might result in relatively low genetic

variation of this fungus under low pressure of environmental factors and fungicide application in the south Australian temperate zone. This is in agreement with other reports that very few fertile cultures have been recognised in newly characterised species of other fungi, such as *Fusarium* (Zeller et al., 2003, Walsh et al., 2010).

Another possible reason for the lack of production of the teleomorph of *P. koolunga* in this study is that the environmental conditions needed (Trapero-Casas & Kaiser, 1992) by this fungus for sexual reproduction were not provided. Moore-Landecker (1992) reported that nutrients such as; carbon and nitrogen sources and vitamins, minerals, fatty acids, pH, temperature, light, water and atmospheric conditions are environmental factors that influence initiation and development of fungal ascocarps. For example, she reported that the pH for production of ascocarps of various fungi ranges from 3 to 9.5, and temperature varies between 5 and 31°C. Also, the response of ascomycete fungi to light quality for production of sexual structures has been reviewed (Moore-Landecker, 1992) and it was concluded that some fungi responded to UV light (230-320 nm), some to near UV and blue light, others to green, yellow and red and, finally, some fungi need far-red light (more than 740 nm). To enhance the possibility of initiation of sexual reproduction of *P. koolunga*, a wide range of environmental factors and nutrients reported for closely related species of ascomycetes comprising different growth media, temperatures and light conditions were examined in this study. Failure to produce pseudothecia in these experiments may indicate that this fungus needs a special treatment or nutrient for sexual reproduction. For example, Kent et al. (2008) found that copper is an essential element for initiation of sexual reproduction by *Cryptococcus neoformans*.

Before further research is undertaken, the mating type of all isolates of *P. koolunga* from different locations in South Australia, Victoria and Western Australia (Tran et al., 2014) must be determined by specific PCR using the primers of Woudenberg et al. (2012) as was conducted by Lichtenzveig (2013, pers. comm.). After that, crossing compatible mating types in pairs would be desirable to enhance the chance of production of pseudothecia of this fungus in laboratory conditions.

Chapter 6

General discussion

General discussion

This research has provided new information on the biology and epidemiology of ascochyta blight on field pea caused by *Phoma koolunga*. A better understanding of the disease cycle was achieved by research on survival of the fungus on plant stubble, seed infection and transmission to seedlings, seed treatment, genotypes response to inoculation and finally the possibility of teleomorph formation in controlled conditions. This study revealed that this fungus survived for longer on field pea stubble placed on the soil surface than buried in soil and that pseudosclerotia survived in potted soil for up to 14 months. Seed harvested from naturally infected plants from several locations in South Australia and Victoria was infected by *P. koolunga*, and the pathogen was transmitted from artificially infected seed to the resulting seedlings with 98% frequency. The effect of four soil temperatures on disease transmission from seed to seedling also was investigated and revealed that disease on seedlings that emerged at the coldest soil temperature, 8 °C, was more severe than at warmer temperatures. Evaluation of efficacy of six fungicides for seed treatment in controlled conditions showed that P-Pickel T® was more effective than Sumisclex® and Jockey Stayer® ($P < 0.05$) and as effective as the remaining three fungicides in reducing disease severity. The field pea genotypes tested in controlled conditions varied in response to inoculation with one isolate of *P. koolunga*. The sexual state of this fungus failed to form in the conditions tested here. Atypical rhizoid-like colonies of *P. koolunga* that formed in sectors from typical colonies or were isolated from field pea seeds plated on nutrient media, did not produce pycnidiospores and showed less virulence than typical colonies when inoculated on field pea seedlings. Modification of the disease cycle based on the results of this study could help to

formulate improved management strategies for this disease, as will be discussed below.

Survival and infectivity of inoculum of *P. koolunga* on stubble decreased over time, as the fungus on stubble that had been buried or left on the soil surface was not pathogenic after 9 and 11 months, respectively, and after 6 and 5 months such stubble did not cause more lesions on field pea seedlings inoculated with than water control in a bioassay ($P < 0.05$). Davidson et al. (1999) reported that field pea stubble infested with *D. pinodes* and *P. medicaginis* var. *pinodella* was highly infectious for the first 4 months post-burial in soil or when it remained on the soil surface, regardless of the soil moisture, and was able to cause crown lesions on field pea seedlings. The result of this research suggest that field pea stubble may play a role in the longer term survival of *P. koolunga*, especially if it remains on the soil surface after grain harvest, as is standard practice in the zero tillage farming system which is currently being used by many farmers in Australia. The main approach to control ascochyta blight due to *D. pinodes* is delayed sowing or burying stubble to escape the peak of ascospore release from stubble (Salam et al., 2011c, Davidson et al., 2013), but this study has not identified ascospores of *P. koolunga*, hence delayed sowing may not be suitable for this particular pathogen. However, the longer survival of *P. koolunga* on stubble compared to *D. pinodes* may suggest that the rotation interval between pea crops is important to reduce inoculum loads. Further research should be undertaken to explore the survival of *P. koolunga* in infested stubble in low, medium and high rainfall zones in Australia (Davidson et al., 2011). This would allow examination of the survival of the fungus on stubble at different ages left on

or buried in soils of different texture, pH and mycobiota and inform understanding of rotation intervals for management of *P. koolunga*.

Persistent structures such as pseudosclerotia or sclerotia, which form on plant materials or in soil, allow extensive longevity and infectivity of several fungi on the next season's hosts compared to other fungal structures *viz.* pycnidia or conidia (Blakeman & Hornby, 1966, Dickinson & Sheridan, 1968). This research confirmed that pseudosclerotia of *P. koolunga* could persist in soil for longer periods than other structures, such as mycelia or pycnidia on plant stubble, and also had the potential to infect the root system of field pea plants in the following seasons. It is still unknown whether or not pseudosclerotia are produced in natural conditions in soil or on plant materials and, if they are, for how long they might survive in nature, particularly in summer between cropping season in Australia. Should pseudosclerotia be produced in nature, rotation interval between field pea crops might need to be reviewed to avoid infection from pseudosclerotia surviving in the soil. Again, if pseudosclerotia are part of the disease cycle, further research on the formation of these structures in soil and on plant materials in field conditions is required to understand their possible role in survival and in the epidemiology of the pathogen in field pea crops.

This research showed that low soil temperature increased transmission rate of seed-borne *P. koolunga* to seedlings. In addition, disease on root and crown of seedlings at 8° C was significantly more severe than at warmer temperatures. In contrast, delayed sowing of field pea crops in Australian farming systems has been shown as an effective method to manage ascochyta blight caused by *D. pinodes* (McMurray et al., 2011, Salam et al., 2011c, Davidson et al., 2013), which is in winter (late May, June and July) when soil is cold. Delayed sowing is

usually recommended for high disease risk areas and any negative impact of sowing into cold soil on managing seed-borne inoculum should be carefully considered. A better understanding of disease transmission via infected seed in field conditions in different rainfall cropping zones in Australia may contribute to modifying recommendations of sowing time accordingly.

The incidences of infection of seeds collected from agronomy trials plots sown early and late were the highest and lowest, 2.7 % and 0.67 %, of those tested from the agronomy trials of Davidson (2012), respectively. Weather data for Hart, SA, where Davidson's trials were conducted, showed several rain events totalling 83 mm between the early sowing time and the late sowing time. This amount of rain seem enough to allow the development of more severe ascochyta blight on early sown than on late sown crops, supporting finding by Bretag et al. (1995) that early sown crops are more likely to have more frequent seed infection. Late sowing not only leads to less ascochyta blight on plants in the field (McDonald & Peck, 2009, Salam et al., 2011b, Salam et al., 2011c, Davidson et al., 2013), but also reduced infection of pods and thereafter seed infection in this study. The importance of rainfall for seed infection was also reflected in results for incidence of infection in seed harvested from WA. For example, despite widespread distribution of ascochyta blight on field pea crops from 1984 to 2013 in WA (Salam et al., 2011a, Salam et al., 2011d, Tran et al., 2014), seed from Dalwallinu, Pingrup and Scaddan in WA collected in 2010 had the lowest incidence of infection of seed with any of the ascochyta blight pathogens compared with other states, and *P. koolunga* was not detected. These results can be attributed to the low rainfall recorded for WA in 2010. In the absence of ascospores, which have not yet been observed for *P. koolunga*, infected seed with

high transmission rate to seedlings, as results here showed, may act as a source of primary inoculum to initiate disease in field pea cropping regions in Australia. Examination of seed infection, particularly in seeds harvested from severely diseased fields or in years when weather conditions are conducive for disease development, may help to minimise the risk of introduction or dissemination of the fungus to other fields or regions in the following years.

P. koolunga is not widely distributed in states of Australia other than SA, Vic. (Davidson et al., 2012) and, recently, WA, where Tran et al. (2014) reported this fungus from field pea plants in different growing areas in 2012, but not from samples collected in 2010 or earlier. This seemingly rapid and widespread distribution of *P. koolunga* to cropping areas in WA could have been via infected seeds, perhaps from SA, as the transfer of seeds harvested from SA or Vic. to WA occurs in some years, especially for new genotypes (Margetts, K., seednet.com.au, pers. comm., 2014). Even a small incidence of infected seed can introduce a seed-borne fungus to districts which are ascochyta blight-free (Gossen et al., 2010). Therefore, the best approach to prevent long distance spread of *P. koolunga* is to use healthy seed or to treat seed with appropriate fungicides before sowing. The results of this research indicated that fungicides such as P-Pickel T® or Thiram® can be used to minimise seed to seedling transmission of *P. koolunga*. These fungicides also are effective against other seed-borne ascochyta blight pathogens (Hawthorne et al., 2010). Further evaluation of the efficacy of seed dressing in field conditions is required to validate the results of this study. Future research on seed treatment to reduce seed-borne transmission of this pathogen to seedlings would provide more information with which to calculate the benefit-cost ratio of application of fungicides in Australia. Furthermore, the possible benefits of seed

dressing, particularly where seeds are sown late into cold soil, should be investigated.

Some field pea genotypes, such as PBA Wharton, newly released in Australia, the breeding line WAPEA2211, and genotypes Kaspa, PBA Twilight and PBA Oura were less susceptible to this fungus when inoculated by spraying with conidia of *P. koolunga* in controlled conditions. Also, aggressiveness of the isolates of *P. koolunga* examined in this research differed based on % leaf area diseased during the first 2 week post-inoculation. Further assessment of these genotypes in field conditions in different rainfall growing regions, with early to late time of sowing, is necessary to evaluate the effect of environmental factors on response to *P. koolunga*. Evaluation of the variability in aggressiveness of a wide range of isolates of *P. koolunga* collected from representative cropping areas in SA, Vic. and WA is also recommended. As *P. koolunga* has become established in many cropping areas in SA and WA in recent years (Davidson et al., 2012, Tran et al., 2014), the development of genotypes of field pea resistant to *P. koolunga* and other ascochyta blight pathogens is warranted.

Atypical colonies from sectors in mycelial cultures or from plant materials such as seed were a common feature of *P. koolunga*. The formation of pycnidium–like structures but lack of pycnidiospores makes it difficult to identify this as *P. koolunga* in routine studies. Therefore, using oat meal agar as a base medium for isolation or subculturing of this fungus or the development of a culture medium which encourages normal growth of this fungus would help researchers to identify this species accurately. Appearance of small lesions similar to ascochyta blight symptoms on field pea plants inoculated with atypical cultures of *P. koolunga* in controlled conditions highlights the question of whether or not

this form of the fungus and these symptoms appear in field conditions. Further research on atypical forms of *P. koolunga* would help to improve our understanding of this fungus. As DNA testing confirmed these atypical cultures to be *P. koolunga*, comparing these colonies with typical isolates in terms of other genetic features is warranted.

As attempts to initiate formation of the teleomorph of *P. koolunga* failed to yield pseudothecia, despite pairing all possible combinations of all available isolates of this fungus, conditions contributing to formation of the sexual stage have yet to be determined. Finding suitable environmental conditions and growth medium for initiation of pseudothecia with examination of several combinations of both mating types from SA and WA isolates could lead to production of these structures. As this fungus is heterothallic (Lichtensveig, J., pers. comm. 2013), it is crucial to know to which mating type each isolate belongs and whether it is female fertile. As this research showed, *P. koolunga* is seed-borne, therefore, seed has potential to disseminate the fungus, which can help movement of mating type isolates to other field pea cropping areas and increase the chance of crossing compatible mating types and subsequently increase the chance of formation of teleomorph of this fungus in nature. Once the sexual state of the fungus is discovered, investigation of the optimum conditions for pseudothecium formation in controlled conditions as well as field situations will be required. Subsequent determination of a distribution map of compatible mating types in field pea growing areas in SA, Vic. and WA would help to estimate the risk of ascospore production and consequently disease initiation from ascospores. These ascospores not only may act as primary inoculum to initiate ascochyta blight, but also can distribute new combinations of genes which bring possible new virulent

pathotypes to hosts resistant to fungicides. Consequently, disease control may need revision according to possible ascospores production.

Based on the findings of this study, in the current conditions in SA, seed-borne infection and infested field pea stubble should be considered as the primary sources of inoculum in the disease cycle of ascochyta blight caused by *P. koolunga*. In recent decades, many field pea growers in SA have adopted minimal tillage to minimise soil erosion and to retain soil moisture. As a result, infested stubble remaining on the soil surface is likely to be one of the most important sources of primary inoculum of *P. koolunga* as well as other pathogens of this disease complex. If the sexual state of *P. koolunga* is found, then the disease cycle will need to be modified in accordance with ascospore production, and the sowing time adjusted based on the time of peak release of ascospores from stubble.

In addition to ascospores, infested plant stubble and infected seeds are important not only in the spread and survival of the pathogen, but they can also distribute compatible mating types of the fungus and increase the chance of the development of the sexual state in nature and sexual reproduction. Again, this will increase the risk of recombination of virulence factors and other traits and may result in the loss of the small levels of resistance in current field pea genotypes.

In conclusion, in years with weather conditions favourable for development of ascochyta blight caused by *P. koolunga*, control of disease should be expanded to consider all aspects of the disease cycle to prevent significant disease progress and subsequently serious economic losses. An integrated strategy involving removal or burial of stubble, sowing healthy seed and/or dressing seed with appropriate fungicides, sowing genotypes with reduced susceptibility, delayed

sowing and application of limited foliar fungicides could facilitate better management of this disease.

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