Epidemiology and management of cercospora leaf spot (*Cercospora zonata*) of faba beans (*Vicia faba*)

by

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ABSTRACT

The disease cercospora leaf spot (CLS), caused by the fungus *Cercospora zonata*, has affected faba bean (*Vicia faba*) production regions in southern Australian in recent years. This study provides new information on the prevalence and significance of the disease and the factors that affect severity.

Temperature, wetness period, plant maturity, pathogen variability and inoculum concentration all influenced infection of faba bean by *C. zonata* in a controlled environment. Disease severity was positively correlated (R^2=0.83 P<0.001) with wet-degree hours (DH_w) and premature defoliation (40-50%) of the lower canopy, which was most severe when the pathogen was inoculated at the mid- to late-vegetative crop growth stages. Pathogenicity tests showed that 29 isolates of *C. zonata* collected from 1999 to 2008 varied in aggressiveness; this was not related to geographical origin of isolates or growth rate *in vitro*, but isolates collected from 2005 to 2008 were more aggressive than those collected in the period 1999-2004.

The temporal and spatial dynamics of the disease on susceptible and resistant genotypes of faba bean were examined. A strong association between the incidence and severity of CLS and soil-borne inoculum was established using comparative analyses of disease on plants in soil sown with faba bean every 3 years since 1997 and in adjacent soil with no history of cultivation of faba bean. Spatial patterns of disease development showed that inoculum spread primarily over short distances during the early stages of CLS epidemics, though dispersal of 4 to 16 m from the infested soil was observed. Non-linear regression using a logistic model described disease development over time on susceptible plants in soil with *in situ* inoculum, whereas an exponential model best described disease gradient with distance from the inoculum source and disease development on resistant plants. There was a positive relationship (R^2=0.93, P<0.05) between disease severity on susceptible plants grown

in soil with infested residue on the surface and the amount of DNA of *C. zonata* detected in the soil. When residues were removed from the soil surface, or depleted rapidly through grazing, the infectivity of soil and the amount of DNA of *C. zonata* detected were significantly less than for soil with residue remaining on the surface. *C. zonata* survived in soil, on infested residue or as fungal propagules in the soil profile, and remained infective for at least 30 months.

The distribution and occurrence, host range and management of CLS of faba bean in southern Australia were studied. *C. zonata* infected narbon bean, lentil and vetch but did not infect pea, chickpea, lathyrus, lupin or canola. A disease survey of 100 commercial faba bean crops in southern Australia showed that CLS was endemic to all districts examined, observed in 87% of crops. Disease severity varied in all districts but was most severe in crops in the south-east of South Australia. Disease incidence and severity were highest in fields planted with faba bean in short rotations (1-4 years) and decreased (R^2=0.13, P=0.006) as the interval between faba bean crops increased. Severity also appeared to be influenced by faba bean residue remaining from the previous year in adjacent fields. CLS manifested as severe lesions on foliage and extensive defoliation, resulting in a 7% reduction in yield in field experiments. Applications of carbendazim, tebuconazole, chlorothalonil and triadimefon significantly reduced CLS severity compared with untreated controls and a single application of either carbendazim or tebuconazole prior to disease onset was identified as an economical application strategy for control of the disease.

A rapid screening technique was developed to identify resistance to *C. zonata* in faba bean genotypes in a controlled environment. All faba bean cultivars commercially available to the Australian industry were susceptible to the disease. The mode of inheritance of resistance to *C. zonata* was determined to be monogenic dominant and this has allowed a relatively simple pathway by which sources of resistance identified in this study can be transferred to adapted faba bean genotypes available to the southern Australian industry.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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

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The following authors agree that the statement of the contributions of jointly authored papers accurately describes their contribution to research manuscripts 1, 2, 3 and 4 and give consent to their inclusion in this thesis.

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This thesis is dedicated to the memory of my father,

Barrington Litchfield Kimber

1936 – 1995

So rarely are great and good the same man.

If we are to go forward, we must first go back and rediscover those precious values - that all reality hinges on moral foundations and that all reality has spiritual control.

Dr Martin Luther King Jr.

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ABBREVIATIONS

ANOVA	analysis of variance
AWS	automatic weather station
BSA	bean seed agar
CA	carrot agar
CER	controlled environment room
CJA	carrot juice agar
CLDA	carrot leaf decoction agar
CLPA	carrot leaf pulp agar
CLS	cercospora leaf spot
CMA	cornmeal agar
CRB	completely randomised block
CSIRO	Commonwealth Scientific and Industrial Research Organisation
cv.	cultivar
cvs	cultivars
DAI	days after inoculation
DAS	days after sowing
Diam	diameter
DNA	deoxyribonucleic acid
GS	growth stage
ITS	internal transcribed spacer
LAD	leaf area diseased
LPLA	loss of photosynthetic leaf area
NSW	New South Wales
NUV	near ultraviolet
PCR	polymerase chain reaction
PDA	potato dextrose agar (full strength)
RCBD	randomised complete block design
RDTS	Root Disease Testing Service
RO	reverse osmosis
SA	South Australia
SARDI	South Australian Research and Development Institute
V8A	V8 juice agar
V8B	V8 juice Broth
Vic.	Victoria
WA	Western Australia
WAS	weeks after sowing

Chapter 1.

Introduction

INTRODUCTION

Cercospora leaf spot (CLS) of faba bean (*Vicia faba* L.) is caused by the fungus *Cercospora zonata* Winter. This foliar disease is commonly identified on faba bean but it is not reported as a major concern to production and little research on the disease has been published, the most recent reviews were published over 50 years ago (Woodward 1932; Yu 1947). Faba beans are an important export crop in Australia, positioned as the world's fourth largest producer (204,000 t) and second largest exporter of faba beans (139,000 t) (FAO, 2011). The majority of the Australian area planted to the crop (115,000 ha) is located in the Mediterranean-type environments of southern Australia, where increased cropping frequency has resulted in the area doubling during the last 15 years (ABARES, 2011). CLS appears to have increased in prevalence and severity in commercial crops since 2004, while no such increase has been reported in other countries where faba bean is commonly grown. The reasons for this increase are not apparent and factors that influence the development and severity of CLS on faba bean are poorly understood.

Several other species of *Cercospora* are recognised as major economic concerns to production of a variety of crop plants in many parts of the world (Smith & Littrell 1980; Latterell & Rossi 1983; Barbetti, 1991; Shane & Teng 1992). In general, management of these diseases utilises resistance incorporated into adapted genotypes or strategic foliar applications of effective fungicides on susceptible cultivars (Ward et al., 1997; Khan & Smith, 2005; Galloway, 2008). Both of these methods require extensive knowledge of the host, the pathogen and conditions that favour the disease (Barbetti & Nichols 1994; Windels et al., 1998; Wolf & Verreet 2002). The impact of CLS on faba bean yield is unknown, and there are no known reports of phenotypic characterisation of germplasm collections of *V. faba* to CLS, or of fungicide efficacy that can assist the industry in disease management. Furthermore, the characteristics of CLS are often mistaken for symptoms of other major

fungal diseases affecting faba bean, leading to incorrect diagnosis. This has affected disease management decisions and accurate appraisal on the incidence of the disease in commercial crops grown in southern Australia.

C. zonata is reported to have a relatively limited host range, predominantly confined to *Vicia* species (Williams, 1987). The pathogen is presumed to persist in crop residue, either as dormant stromatic mycelium or as clusters of conidiophores (fascicles) which remain on the soil surface and, in conducive conditions in subsequent seasons, conidia are dispersed by wind or rain-splash to infect emerging faba bean plants (Yu, 1947; Walker, 1952; Williams, 1987). The current recommendation for Australian growers is to allow a break of approximately 4 years between faba beans crops within one field, by which time residues harbouring survival structures of other major pathogens of faba bean (*Ascochyta fabae*, *Botrytis fabae*) have decomposed (Wallen & Galway, 1977; Dyke & Prew, 1983). Research conducted on other *Cercospora* species shows that pathogen survival on infested residues varies from several months to several years when residues are retained on the soil surface, but that inoculum levels can be reduced using various cultural practices (Nagel, 1938; Kilpatrick, 1956; Payne & Waldron, 1983; Cooperman et al., 1986; de Nazareno et al., 1992). However, the life cycle of *C. zonata* is poorly understood and this research was intended to provide new information on the sources of inoculum and epidemiological factors affecting disease development, and survival and dispersal of the pathogen. The project was initiated with support from the Grains Research and Development Corporation (GRDC), with the expectation that outcomes would contribute to the development of improved disease management practices.

The research described in this thesis was undertaken to i) determine the prevalence of CLS in the field and its significance to the Australian faba bean industry, ii) investigate factors that affect the development of CLS on faba bean, iii) assess germplasm in the collection of the Australian faba bean breeding program for genotypes that could be used in

the development of cultivars resistant to CLS, and iv) develop management strategies and recommendations for control of the disease.

Chapter 2.

Literature review

LITERATURE REVIEW

2.1 INTRODUCTION

Cercospora leaf spot (CLS), causal agent *Cercospora zonata* Winter, is a common foliar fungal disease of faba bean (*Vicia faba*). Until recently, it has not posed a sufficient threat to the Australian industry to warrant thorough investigation. The prevalence and severity of CLS increased in both research trials and commercial crops in southern Australia from 2004 and the reason for this increase is not clear. Detailed knowledge of *C. zonata* and its effect on faba beans is limited and the impact of the disease on yield and possible methods of control need elucidation.

Symptoms of CLS are easily confused with those of ascochyta blight (*Ascochyta fabae*) and chocolate spot (*Botrytis fabae*), two of the major diseases of faba beans. Many growers, in particular, are not familiar with its symptoms and consequently, misidentification may lead them to apply costly fungicide sprays that have not been proved effective, or necessary, for control of CLS. Those growers familiar with the disease have expressed concern at the increased incidence of this disease and questioned whether current control strategies, implemented for other diseases in faba beans, may be used to effectively control CLS (pers. com., Wayne Hawthorne, 2005). An initial step in addressing this question was to determine the impact of the disease on faba bean yields.

Other species of *Cercospora* have been recognised as damaging foliar pathogens in other crops, for example CLS of sugar beet. This review will draw comparisons and principles from studies conducted on these related species, and where appropriate, use them to build an understanding of principles that may be applicable to CLS in faba beans.

A better understanding of the epidemiology of CLS in faba beans, its impact on yield and the exploration of host resistance will assist in the development of management strategies.

This project will provide information about the factors that have contributed to the emergence of this disease and determine the significance of CLS to the Australian faba bean industry.

For this review, literature on CLS of faba bean and relevant aspects in some comparable studies on related pathogens was examined. It was predominantly compiled in 2005. Relevant research articles and discussion papers published since that time are included in the discussion sections of each chapter and in the general discussion.

2.2 HISTORY OF FABA BEANS AND THE AUSTRALIAN INDUSTRY

Faba beans, *Vicia faba* L, belong to the Leguminosae. The phenotypic description of faba bean is a cross-pollinated, erect, simple stemmed annual, normally 50 – 200 cm in height, with one or more basal branches. Leaves are alternate and pinnate with two or more oval leaflets. Short axillary racemes form at flowering nodes, and seeds are produced in pods that vary in size with the number and size of seeds (Dennis, 1991). Within the *eu-faba* classification, there are three botanical varieties; the large-seeded var. *major*, the medium-seeded var. *equina* and the small-seeded var. *minor* (Hawtin & Hebblethwaite, 1983). However, seed size or length are not always agreed on as the most suitable characteristics for classification of these sub-groups, with seed testa colour, seed hilum colour and melanin spot in flowering petals also used (Higgins et al., 1981). *Vicia faba* var. *equina* and *minor* are commonly referred to as field beans in the United Kingdom, Europe and Australia, and *V. faba* var. *major* is also known also as broad bean. The use of common names to identify this species can cause confusion. In the United States of America, *Phaseolus vulgaris* is known as the field bean, and this has been used frequently in scientific literature (Hawtin & Hebblethwaite, 1983). However, most recently, the name faba bean is the most universally adopted term to denote all varieties of *V. faba* and is the name accepted for this review.

The origin of faba beans is not as clear as it is for many of the main pulses such as lentils, peas and chickpeas; most likely due to the confusion in classification of the species and sub species. Only recently, well-preserved seeds of *V. faba* were found at Tell el-Kerkh, in north-west Syria. This represents the earliest archaeobotanical finds of the species, and suggests a date of origin for faba beans *ca* 8,000 B.C. (Tanno & Willcox, 2005). Previously, the origin of faba beans was thought to have been identified in a pre-pottery Neolithic layer in southern Jordan, dated *ca* 6500 B.C., though these were possibly *V. narbonensis* (Renfrew, 1969). Likewise, a recording of the oldest seeds claimed to be faba bean found at Jericho (Hopf, 1969), dated *ca* 6250 B.C., comprised very small seeds and, again it has been proposed that this is a related species (Hawtin & Hebblethwaite, 1983). Nevertheless, assuming that *V. faba* and other related species evolved from a common ancestral stock, it is likely that the cultivated faba bean was domesticated in Neolithic times in the Mediterranean Basin (Tanno & Willcox, 2005) and later spread to Spain, Portugal and Eastern Europe. Ladizinsky (1975), however, favours domestication in central Asia and introduction into Europe with invading tribes. Faba beans have been recorded in China and India for over a thousand years and were introduced into North and South America and Australasia with the first European migration. Vavilov, a germplasm curator from Soviet Russia, supported the latter theory after the discovery of a primitive type of faba bean with small pods and seeds at the intersection of the Himalaya and Hindu Kush (Lang et al., 1993). Given the numerous assertions, and the absence of a commonly accepted wild progenitor, the origin of faba beans is likely to remain uncertain. In addition, no successful crosses have been made between *V. faba* and any other *Vicia* species (Lawes et al., 1983). Consequently, this emphasises the importance of international germplasm collections of *V. faba* that offer valuable sources of genetic variability.

Today, faba beans are grown in temperate and subtropical regions throughout the world. The major producers of faba beans in 2009 (>50,000 tonnes), in approximate

descending order, are China (dominating), France, Egypt, Australia, United Kingdom, Sudan, Italy, Peru and Tunisia (FAO, 2011). The major importers are Egypt, Sudan, Italy, Spain and, to a lesser extent, several countries in the Middle East (FAO, 2011).

In Australia, the faba bean industry effectively commenced with the release of an adapted cultivar (Fiord) by the University of Adelaide, South Australia, in 1980 (Knight, 1994). The breeding program had introduced germplasm from many countries and it soon became evident that the best adapted and highest yielding material originated from the Mediterranean region, which has an environment similar to southern Australia (Siddique et al., 1997). Before this time, the industry constituted a small and inconsistent area sown to faba beans across various states from the 1920s onwards, with cultivars imported from the United Kingdom (Day & Hawthorne, 2004). A formal effort to breed improved cultivars of faba bean, adapted to Australian environments, commenced at the University of Adelaide in 1976. The Australian faba bean industry has strengthened considerably over its 30-year history and now services the high rainfall cropping zones for southern Australia, where approximately 95% of faba beans are grown, 85% of this in South Australia and Victoria. In 2008, Australia was the 4th largest producer and the second largest exporter of faba beans in the world, the latter in close competition with France (FAO, 2011). The area planted to faba bean has steadily increased in Australia since the mid-80s and peaked in 2000, with 206,000 ha planted, after which a series of droughts resulted in a decrease in the area planted to the crop (Figure 2.1)

Faba beans are grown as a winter crop in Australia, typically planted in late autumn (May) and harvested in early summer (December). Problems such as foliar diseases, inconsistent seed quality and regional adaptation have all affected the development of the industry. In addition, the incorporation of other legume crops (lentils and chickpeas) in rotations and the increase in area sown to canola, have contributed to the restriction or decline in area sown to beans in southern Australia (Day & Hawthorne, 2004). Diseases have long

been a major issue with the crop, with several epidemics of ascochyta blight and chocolate spot having occurred throughout the growth of the industry in Australia. Nevertheless, growers have persisted with faba beans as a break-crop, particularly after its potential was recognised in rotation with cereals (Laurence, 1979), due to its ability to fix nitrogen through association with *Rhizobium* bacteria. In the UK, the effect of a faba bean crop on the yield of a following barley crop was estimated to be equivalent to 40 kg/ha of nitrogen fertiliser (Prew & Dyke, 1979). In Australian trials, net nitrogen balance remaining in soils after faba beans was up to 94 kg N/ha (Schwenke et al., 1998). As the Australian industry has expanded, further benefits have been identified including a reduction in root diseases and increased yield of cereals following pulse crops and opportunities to control grassy weeds, which are difficult or expensive to control in cereals (Brouwer, 1994). Faba beans and other pulse crops now have a positive role in the predominantly cereal-based cropping system of southern Australia, with an increasing recognition of their importance in cropping rotations.

NOTE:
This figure is included on page 13 of the print copy of the thesis held in the University of Adelaide Library.

Figure 2.1: Area planted with faba bean in Australia from 1986 to 2010 (ABARES, 2011).

Australian faba beans are marketed for either domestic stockfeed or human consumption, with approximately an equal split between the two markets (Siddique & Sykes, 1997). However, recent strategies to expand the export market for human consumption are providing new momentum to the industry and as a result, the crop is more readily included within rotations in southern Australia. Though not consumed to any significant level in the domestic market, faba beans comprise an essential component of the human diet in many countries, particularly the Eastern Mediterranean, the Middle East and Asian regions (Simpson, 1983; Hawtin, 1981). On average, the daily consumption of faba beans in Egypt is around 2000 tonnes (Tadros, 2004). Faba beans offer an economic and easily accessible alternative to meat as a source of protein, with approximately 100 g of cooked faba beans meeting the daily requirements of essential amino acids for an average human (Ali et al., 1982).

2.3 HISTORY AND SIGNIFICANCE OF CERCOSPORA LEAF SPOT

CLS, sometimes referred to as faba bean zonata spot, is caused by a fungus that attacks the foliage of faba beans. The disease is relatively common on faba beans in most regions where they are grown, but its economic significance is not understood. There appear to have been only two specific yet brief reviews on this disease in the literature, one by R.C. Woodward (1932) in response to the first reported occurrence of the disease in the United Kingdom in 1927 and another by T.F. Yu in China (1947). Both authors stated, at that time, the disease had been reported in China, Bohemia, France, Japan, England, Cyprus and Italy.

Ascochyta blight and chocolate spot are the main diseases that can reduce yield and quality of faba beans in southern Australia. CLS is present throughout most cropping regions where faba beans are grown but no formal record of incursion into Australia appears to be documented. The disease has been labelled an ‘occasional’ problem in southern Australia, often observed in commercial crops in particularly wet growing seasons (Anonymous, 1996),

but has typically been over-shadowed by ascochyta blight. Nevertheless, sporadic yet severe disease has been observed in faba bean research trials and commercial crops in the past, particularly in the South-East of South Australia (pers. com., Wayne Hawthorne, 2003). However, since 2004, the prevalence of CLS has increased in most growing regions of South Australia, Victoria and Western Australia.

No publications have been found detailing research on CLS in Australia and only a few studies have been reported in other countries, which do not focus on CLS alone. Technical bulletins and advisory sheets issued to industry which address management of foliar diseases in faba beans rarely mention CLS. Limited publications that make comment on the disease simply describe the symptoms. The shortfall in available research on the disease has made addressing growers' concerns difficult.

2.4 THE CAUSAL AGENT AND THE DISEASE

The causal agent of CLS of faba beans is most commonly identified as *Cercospora zonata* Winter, Fungi Imperfecti, belonging to the order Hyphomycetales. The pathogen was first described in the late 1800s, initially by Winter as *C. zonata* in 1883 and later by Fautrey as *C. fabae* in 1890, but the species were later concluded to be be identical (Woodward, 1932). During the early stages of classification, the causal agent was also referred to as *C. viciae* or *Cercosporina fabae*, though the latter was suggested to be incorrect (Chupp, 1954). The life history of the pathogen has not been studied critically. The pathogen is thought to survive between crop seasons as conidiophores or dormant mycelium on infected faba bean debris (Yu, 1947; Walker, 1952) and its method of transmission is presumed to be by air-borne, or rain-splash dispersed, conidia (Williams, 1987).

Cercospora species are a successful group of pathogens, causing damaging leaf spot and blight diseases on a diversity of crop species worldwide. Among the most destructive of

these diseases are; CLS of sugar beet caused by *C. beticola*, grey leaf spot (*C. zeae-maydis*) of corn, purple seed stain (*C. kikuchii*) of soybean, frogeye leaf spot (*C. nicotianae*) of tobacco, and brown eye spot (*C. coffeicola*) of coffee (Daub & Ehrenshaft, 2000). The importance of the *Cercospora* pathogens is attributed not only to their prevalence and widespread distribution, but also the susceptibility of commercial cultivars of some important crops to these diseases (Wang et al., 1998; Windels et al., 1998).

In what is commonly referred to as the *Cercospora* complex over 3,000 named species are listed, including those belonging to the genera *Cercosporella*, *Cercosporidium*, *Pseudocercospora* and *Pseudocercosporella* (Goodwin et al., 2001). Most of the species of *Cercospora* have no known sexual stage, although a *Mycosphaerella* teleomorph has been identified for a few; *Mycosphaerella fijiensis* (*Cercospora fijiensis*) and *Mycosphaerella musicola* (*Cercospora musae*) on banana, and *Mycosphaerella arachidis* (*Cercospora arachidicola*) on peanut (Corlett, 1991). Other associations between *Cercospora* species and *Mycosphaerella* teleomorphs have been reported but not confirmed (Goodwin et al., 2001).

2.4.1 Macroscopic description

Symptoms appear predominantly as red-brown to dark-grey leaf spots (Figure 2.2A) that are subcircular to angular, often concentrically zonate, and frequently display a broad, slightly raised, deep red margin (Figure 2.2B) (Yu, 1947; Walker, 1952). The disease mainly affects leaves, but may also affect stems and pods of faba beans (Lang et al., 1993). Lesions initially form on lower leaves of the seedling, early in the growing season, and gradually progress up the plant if conditions are favourable for disease development. The symptoms are not so characteristic that they are readily distinguished from ascochyta leaf spot (*Ascochyta fabae*) or chocolate spot (*Botrytis fabae*). Lesions may first appear 1-5 mm in diameter but can expand rapidly to 15 mm and coalesce with adjacent lesions (Figure 2.2C) resulting in severe blighting of the entire leaf. These spots may occur on any part of the leaf surface,

including tips and margins, and are not limited by veins (Yu, 1947). Under moist conditions, the fruiting bodies, i.e. conidiophores and conidia, can protrude from any part of the lesion (Figure 2.2D), mostly on the upper surface (Chupp, 1954). Severe infection may result in extensive defoliation.

Yu (1947) stated that *C. zonata* rarely infects petiole, stipule and stem and few reports mention this type of infection. However, red to dark grey and elliptical lesions on stems were described by Lang et al. (1993), though sporulation of the pathogen was not confirmed. Yu (1947) stated that stem lesions are almost indistinguishable from the young lesions produced by *Ascochyta fabae*, and also stated that sporulation of *C. zonata* had not been observed on infected stems.

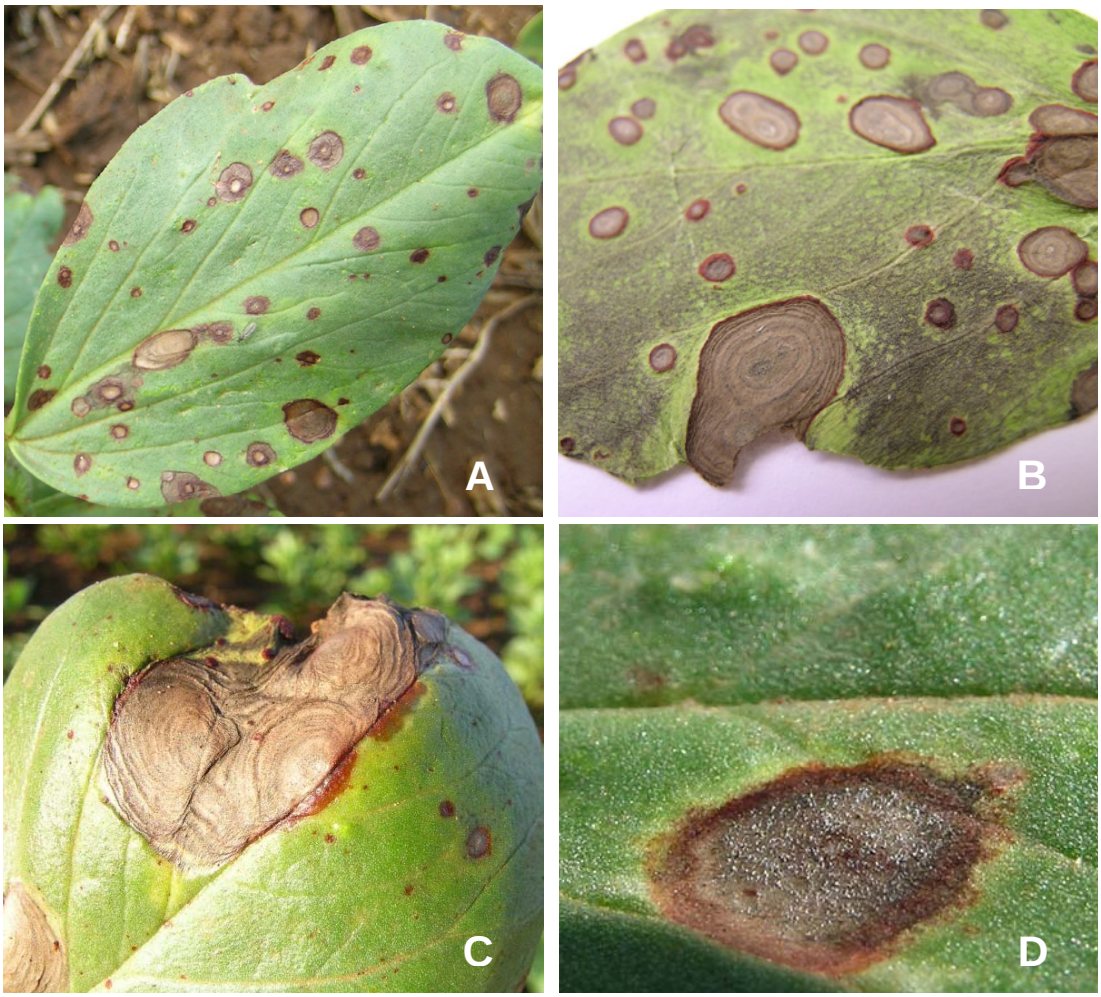

Figure 2.2: Symptoms of Cercospora leaf spot (*C. zonata*) on faba bean: A, Leaf lesions. B, Partially dried faba bean leaf exhibiting distinct zonate lesions. C, Coalesced lesions. D, Sporulating leaf lesion.

2.4.2 Microscopic description

The fruiting bodies of the fungus are normally observed on established leaf lesions in moist environments. They consist of minute bundles (fascicles) of conidiophores (Figure 2.3A) in clusters of 1-12, mostly 3 to 6, emerging from the leaf surface (Yu, 1947). Conidiophores are dark brown to black at the base, paler above, and morphologically different from vegetative hyphae. These conidiophores are dark cells, unbranched, straight or curved, usually septate and are 25-60 µm long and 4-7 µm wide (often wider at the base). The conidiogenous cells are integrated, terminal, rarely bent and the thickened scars have a distinct central pore. Conidia (Figure 2.3B) are colourless, solitary, acicular, often curved, with a truncate base bearing a scar, and a sub-acute apex, 3- to 18-septate and 30-150 µm long, 3.5-5.5 µm wide (Williams, 1987). However, Welles (1924) pointed out that conidia of some species of *Cercospora* often vary in size and septation in different environments.

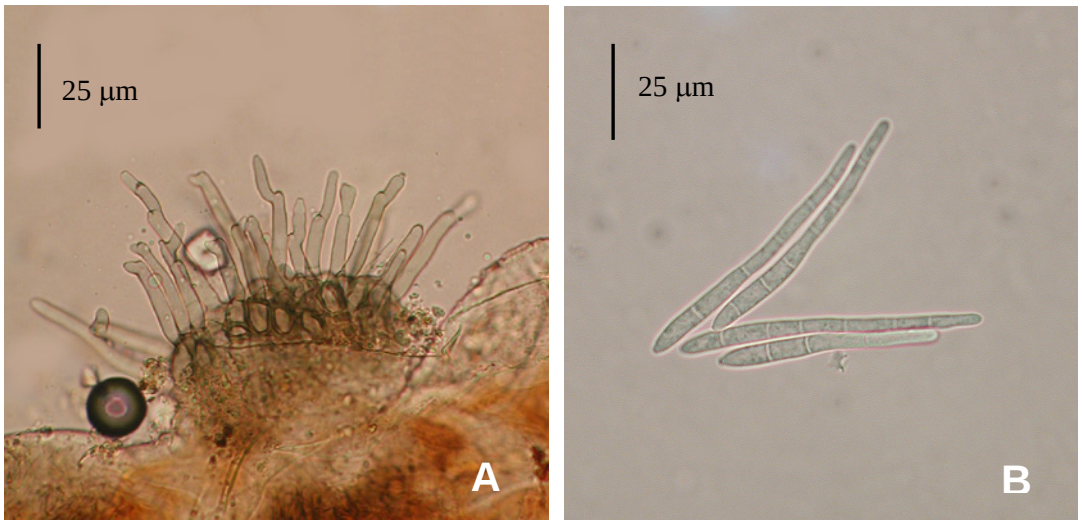

Figure 2.3: Microscopic morphology of *Cercospora zonata*: A, Conidiophores (conidia detached). B, Conidia.

2.4.3 Cultural characteristics

Sporulation of *C. zonata* in artificial culture media has not been reported. Numerous media and culture techniques have been described that induce sporulation in other species of this genus (Nagel, 1934; Cooperman & Jenkins, 1986; Paul & Munkvold, 2005). However,

Yu (1947) stated that attempts to induce sporulation in *C. fabae* (*C. zonata*) using methods described by Nagel (1934) were not successful and most abundant growth was on potato-dextrose agar (PDA). Barbetti (1985) reported poor sporulation of *C. zebrina* despite testing cultural techniques and media recommended to induce sporulation. A subsequent study of *C. zebrina*, the casual agent of cercospora blackstem disease in subterranean clover, reported by Barbetti (1991), described inoculation of this fungus by means of hyphal suspension, which proved an effective method and did not appear to impair infection of the host.

When grown on PDA, the fungus is first white and gradually becomes grey. As growth proceeds, the colony becomes dark grey to black with the centre slightly raised and the outer margins often exhibiting one or two zones (Yu, 1947). Colonies often develop irregular areas of white, dark grey or deep olivaceous green patches on the surface. In old cultures, the mycelium can become dense and compact and often form a rippling effect. Yu (1947) stated that optimum growth, determined by colony expansion rate, was recorded at 25^{o}C with a maximum slightly over 31^{o}C and a minimum at approximately 5^{o}C. Cultures stored at 5^{o}C apparently grew for long periods of time.

2.4.4 Morphological and pathogenic variation

Due to the rarity of useful morphological and physiological characters, the taxonomy of the *Cercospora* complex remains confusing and depends heavily on the host (Goodwin et al., 2001). There is no published research that addresses either morphological or pathogenic variation in *C. zonata*. Chupp (1954) commented on the morphology of a collection of *C. zonata* isolates, stating that comparative studies are difficult as collections have very few fruiting bodies. Nevertheless, recent population studies of *C. zeae-maydis*, the causal agent of grey leaf spot in maize, revealed the presence of two pathotypes, designated group I and II, based predominantly on genetic diversity (Wang et al., 1998). Later studies on this pathogen have shown that different *C. zeae-maydis* populations vary in aggressiveness, which may have

influenced recent epidemics in maize crops in East Africa (Carson et al., 2002; Okori et al., 2004). It is feasible that a shift in aggressiveness within the populations of *C. zonata* could explain the recent increase in prevalence and severity of the disease in faba bean crops in southern Australia, although similar studies would be needed to confirm this suggestion.

Pathogenicity of *C. zonata* to faba beans has been described by Yu (1947), in which faba beans seedlings were inoculated with a water suspension of conidia obtained from washed diseased plant material. Symptoms first appeared 2 to 3 days after inoculation and zonate lesions, typical of field infections, formed after 10 to 15 days. Pathogenicity was demonstrated on non-injured leaves of faba beans and was in contrast to pathogenicity studies conducted by Woodward (1932), who stated that spores of *C. fabae* readily infected leaves of faba beans by way of wounds and that, in artificial conditions, non-injured leaves did not become infected. The conflicting findings reported by these authors reflect the difficulties of working with this pathogen in controlled conditions.

2.4.5 Host specialisation

C. zonata is reported to have a relatively limited host range, confined to *Vicia* species which include *V. faba, V. narbonensis* (narbon bean) and *V. sativa* (vetch). Yu (1947) stated that the fungus was not able to infect the following species of plants: *Dolichus lablab* L., *Glycine max* Merr., *Lathyrus odoratus* L., *Lens esculenta* Moench., *Medicago sativa* L., *Melilotus alba* Desr., *Phaseolus vulgaris* L., *Pisum sativum* L., *P. sativum* var. *arvense* Poir., *Trifolium repens* L., *T. pratense* L., *V. sativa* L., *V. cracca* L., *V. villosa* Roth. and *Vigna sinesis* Endl. However, this is in direct contrast to later studies, in which the pathogen was reported to infect *Lathyrus odoratus* (lathyrus) and *Lens culinaris* (*L. esculenta*) (Chupp, 1954; Williams, 1987). The reasons for this apparent contradiction in the literature cannot be explained, as the methodologies of these studies were not detailed. Vetch and lentils in particular are commonly grown in the cereal belt of southern Australia, as part of rotation

schedules which often include faba beans. The severity of pathogenicity on these alternative hosts is unclear, as is their role in the spread of CLS in faba beans. Spread of *Cercospora* species from alternative hosts has been recorded in the past, such as angular leaf spot (*C. puerariicola*) on kudzu (*Pueraria thunbergiana*) (Weimer & Luttrell, 1948). Extensive studies on host specialisation of *C. zonata* are required to determine the role of alternative hosts in the persistence of the pathogen in cropping systems.

Jones (1944) conducted host specialisation studies on *Cercospora* isolates collected from sweet clover (*Medicago alba*), lucerne (*M. sativa*) and red clover (*Trifolium pratense* L.) and reported that these fungi would infect only the host from which the isolate was derived. However, Berger & Hanson (1963a) found that cross-infection would occur for isolates collected from *Trifolium*, *Medicago* and *Melilotus* spp., though these isolates were generally more pathogenic on the host species from which they were isolated. In studies conducted on *C. zebrina*, the causal agent of cercospora blackstem, a high degree of susceptibility in a number of hosts, namely *Trifoilium hirtum* (rose clover), *M. truncatula* (medic) and *M. sativa* (lucerne) was reported (Barbetti, 1985). This suggested that these pasture species, in addition to *T. subterraneum* cultivars, are also likely to be adversely affected by the spread of the disease in Western Australia.

In South Australia during 2004, *Pseudocercosporella capsellae*, the causal agent of grey leaf spot of canola, was identified in a number of canola crops. This was the first known report of this disease on canola in South Australia (pers. com., Chris Wilmshurst, 2004). An increase in the prevalence of *Cercospora* spp. in South Australia in recent seasons does raise the issue of host specialisation of these pathogens and whether cross-infection combinations may play a part in disease establishment in faba bean fields. It is assumed that these pathogens are specific to the host from which they were isolated, but this remains to be tested. Lartley et al. (2005) detailed cross-infection studies of *Cercospora* spp. with respect to protecting the sugar beet industry in the Midwestern United States of America. Their

research was conducted in response to observations of unusual leaf spots in safflower fields grown in a region where sugar beet is routinely affected by CLS (*C. beticola*). Safflower is known to be susceptible to *C. carthami* but this pathogen had not previously been recorded in the region. Lartley et al. (2005) showed that the lesions on safflower were actually caused by *C. beticola*. Safflower, which was being evaluated as a potential rotation crop with sugar beet was, therefore, identified as an alternative host for the casual agent of CLS in sugar beet, making safflower unsuitable for this purpose.

2.4.6 Production of the phytotoxic metabolite, cercosporin

Many species of *Cercospora* are characterised by the production of a phytotoxic metabolite of polyketide origin, called cercosporin (Assante et al., 1997). Cercosporin is classified as a photosensitiser (Daub, 1982) and represents a defined group of diverse compounds that are activated by visible wavelengths of light, generating activated oxygen species toxic to living cells, a process often referred to as photodynamic action (Yamazaki et al., 1975; Daub & Ehrenshaft, 2000). It is now known that common biological molecules, such as flavins, are photosensitisers and that plants produce many examples of such compounds, such as chlorophyll. A well-documented example is hypericin, produced by St. John's wort (*Hypericum perforatum*), a popular herbal remedy for depression. This potent photosensitiser is also known to be toxic to animals and toxicity in humans upon sun exposure has been reported (Daub & Ehrenshaft, 2000). Because of their broad-spectrum toxicity, photosensitisers are often investigated for use as insecticides, herbicides and medical pharmaceuticals.

Cercosporin has been identified as toxic to plant cells, but production only occurs in the presence of light, with a linear relationship between light intensity and cell death (Daub & Ehrenshaft, 2000). Although this compound has been linked to pathogen virulence, it is not a universal pathogenicity factor because it is not produced by all species of this genus of fungi

(Assante et al., 1997). Production may also be affected by many environmental and nutritional factors, and can be specific to strains or isolates of a single species (Goodwin et al., 2001). Many studies have been conducted that examine production of cercosporin by species of *Cercospora*. Nevertheless, it appears that production of this toxin by *C. zonata* has not been examined, thus no conclusive evidence can be drawn on this aspect of the interaction with faba bean.

2.5 EPIDEMIOLOGY OF CERCOSPORA LEAF SPOT

The development of a disease epidemic on plants depends on several factors relating to the host, the pathogen, the environment and the complex interaction of these factors (Agrios, 1997). The epidemiology of CLS of faba beans has not been described. In some plant pathosystems, which include many *Cercospora* species on various hosts, the pathogen mainly causes discrete lesions on leaves that may remain restricted in size over the life of the host. In contrast, when conditions are favourable, the lesions continue to grow rapidly after their initial appearance, until much of the host is symptomatic. This is known as lesion expansion and is an important component of epidemiology, particularly relevant to the cercospora leaf diseases (Berger, 1977; Berger & Roberts, 1990; Berger et al., 1997). Lesion size and development has been used extensively in pathology studies to assess disease severity and in plant breeding to rank germplasm for resistance to various pathogens (Tivoli et al., 2006). The fundamental factors that influence development of CLS and the potential for severe disease on Australian cultivars of faba bean require elucidation.

2.5.1 Effect of temperature and moisture

The occurrence of CLS in faba beans within southern Australia was reported to be confined to seasons with prolonged free moisture in spring (Anonymous, 1996). However,

recent observations show that the disease has occurred early in the growing season, apparently unimpeded by the cold winter temperatures, and continued to develop through the warmer spring months. The optimal environmental conditions for the disease are not understood, particularly the influence of temperature and moisture. However, principles may be drawn from research on other species of this group of fungi.

Windels et al. (1998) stated that conidia of *C. beticola* are produced most readily at temperatures from 20 to 26 ^{o}C and relatively humidity from 90 to 100%, but ideal conditions for germination and infection occur at 25 to 35 ^{o}C when free water remains on the leaves for at least 8.5 h. However, conidia do not form at temperatures less than 10^{o}C. Studies conducted on CLS of sugar beet (*C. beticola*) showed that spore concentration, temperature and duration of leaf wetness all influenced the pathogen incubation period and disease severity on sugar beet. Wallin & Loonan (1971) reported an increase in leaf spots in the order of 30 to 80 times after periods of 48 or 72 h leaf wetness, respectively, compared to a leaf wetness period of 24 h. This was particularly evident when inoculum concentrations were greater than 2500 spores/ml and temperatures were 24-29^{o}C, compared to 10-18^{o}C.

Warm wet conditions are favourable for many cercospora leaf diseases, as was reported above for CLS of sugar beet and also for cercospora blackstem disease (*C. zebrina*) on subterranean clover (Barbetti, 1985), cercospora grey leaf spot (*C. zeae-maydis*) of maize (Paul & Munkvold, 2005) and cercospora blight (*C. asparagi*) in asparagus (Cooperman & Jenkins, 1986). Optimal temperatures between 22-28^{o}C are reported for these pathogens during infection of their respective hosts. Though these pathogens represent species of *Cercospora*, the optimal temperature for infection appeared considerably different from that of *C. zonata* in faba beans. These studies do not provide an explanation for the rapid development of the disease on faba beans during cold temperatures (5-15^{o}C) often experienced during winter in southern Australia.

2.5.2 Defoliation as an epidemiological component

A secondary component of the epidemiology of CLS on faba beans may be the impact of defoliation on plant growth, particularly on the plant's photosynthetic capacity. Defoliation is a frequent symptom of the host affected by a cercospora leaf disease when conditions are favourable to the pathogen. This area of epidemiology is not well understood, but may be particularly relevant to *C. zonata* on faba beans, as the pathogen predominantly attacks leaves. The main foliar fungal diseases currently affecting faba bean fields in southern Australia, ascochyta blight and chocolate spot, affect stems and pods as frequently as leaves, thus having a more direct impact on plant yield or grain quality.

The effect of defoliation on plant growth was shown in studies of early and late leaf blight (*C. arachidicola* and *Cercosporidium personatum*), and rust (*Puccinia arachidis*) of peanut (*Arachis hypogaea* L.). The control of these foliar diseases in peanuts grown in the tropical monsoonal climate of the Ord River Irrigation Area (Western Australia) is an essential part of cropping strategies (Bell, 1986). Boote et al. (1980) found that peanut infected with *Cercospora* spp. had reduced photosynthetic efficiency, as well as an associated reduction in interception of incident radiation, as a result of defoliation caused by the pathogen. This reduction was due to a reduced efficiency of CO_2 fixation by the remaining leaves as well as to leaf loss. Boote et al. (1980) stated that the top third of the crop canopy intercepted 75 and 58% of the total incident radiation in disease-free and severe-disease conditions, respectively, and suffered proportionally much less leaf loss due to *Cercospora* sp. than the lower two-thirds of the canopy. However, they examined only one cultivar of peanut. Subrahmanyam et al. (1984) evaluated 20 peanut cultivars and found high levels of infection from *Cercospora* sp. caused rapid and severe defoliation and that yield was highly correlated with remaining green leaf material. However, Bell (1986) found that control of all foliar pathogens in peanut resulted in a depression in yield. This statement infers that a certain amount of defoliation may not be detrimental to yield. However, the author

appropriately cautions against the use of foliar pathogens to manipulate canopy characteristics until more is known about the effects of pathogens on photosynthetic capacity of remaining leaves and the extent to which an epidemic is allowed to develop before control measures would then become ineffective.

2.5.3 Pathogen survival

Faba bean residue, which remains in the field after each season, provides an important source of inoculum for several diseases common to faba bean in Southern Australia. CLS is also presumed to persist in crop residue, as dormant mycelium, remaining on the soil surface and to infect emerging faba bean plants in subsequent seasons (Walker, 1952). Yu (1947) demonstrated the 'overwintering' survival phase in a basic field study in 1929, possibly the only published study of survival of this pathogen. Yu (1947) showed that diseased bean leaves, which had been buried 1 inch (2.5 cm) below the soil surface between wire screens for 8 months, were effective as an inoculum source of the disease. The decayed leaves, along with any adhering soil, were placed on the soil surface where bean seeds had been sown and exposed to high humidity for 2 days before being set in a cool greenhouse. After a few weeks, symptoms of CLS formed on the seedlings. In addition, Yu (1947) was able to collect fresh conidia of *C. fabae* (*C. zonata*) in the supernatant when decayed leaves and soil were soaked for about 20 min. This suspension of conidia was also shown to cause disease when sprayed onto bean seedlings. The author further stated that microscopic examination of the decayed material revealed that either conidiophore clusters (presumably fascicles) or stromatic mycelium remained alive throughout the winter, and that they must produce fresh conidia when conditions become favourable. This experiment was reportedly repeated, with the same result, 4 years later (Yu, 1947).

However, the duration of survival and the environmental conditions in which the pathogen may persist are not known. There do not appear to be any reports of research in

Mediterranean climates, where the pathogen must survive over summer, exposed to heat with little free moisture. The current rotation recommendation for fields returning to faba beans is approximately 4 years, after which crop residues and, consequently, survival structures of pathogens such as *A. fabae*, typically have decayed after burial and no longer represent a significant source of inoculum (Wallen & Galway, 1977; Dyke & Prew, 1983). However, a high incidence of CLS has been observed in research trials and commercial crops of faba beans in Southern Australia that have returned to faba beans after a 3 year break, suggesting that a significant amount of *C. zonata* inoculum might survive beyond 4 years.

Survival on crop debris is the most important source of inoculum for many species of *Cercospora*. Two such examples are *C. beticola* and *C. zeae-maydis*. *C. beticola* survives mainly as stromata on infected sugar beet leaves, although survival on beet seeds and several common weed hosts, such as redroot pigweed, lamb's-quarters, mallow and bindweed, also serves as minor sources of inoculum (Windels et al., 1998). In comparison, survival of *C. zeae-maydis* on maize is confined to infested crop debris (Payne & Waldron, 1983).

2.5.4 Disease spread

C. zonata is presumed to be spread by air-borne, or rain-splashed dispersed, conidia (Williams, 1987). However, the patterns of dispersal and the factors that influence dissemination of the pathogen are not well understood. Therefore, studies conducted on related species were examined as a guide to potential dispersal mechanisms of *C. zonata*.

The spread of *C. zeae-maydis* begins with production of conidia in lesions on crop debris remaining on the soil surface (Payne & Waldron, 1983). These conidia, known to be the primary inoculum causing grey leaf spot, are wind-dispersed. On contact with a susceptible host, stomatal penetration, vegetative growth and production of new conidia from stromata within the developing leaf lesion require 2 to 4 weeks of favourable conditions (Ward et al., 1999). These new conidia may be spread by wind or rain to other leaves and

plants, causing secondary infections during the growing season. Conidia of *Cercospora* species are borne on erect conidiophores above the leaf surface and protrude into the layer of turbulent air above the leaf surface (Meredith, 1973). The passively detached spores are then dispersed by wind (Lapaire & Dunkle, 2003).

Berger (1969) reported the use of spore trapping to permit accurate predictions of dispersal of spores of *Cercospora apii* on celery. Spore production and release increased progressively with each successive night of 8 h or more where relatively humidity (RH) was near 100% and temperatures between 15-30^{o}C. However, when night-time temperatures fell below 15^{o}C, negligible spores were trapped, regardless of RH. After such a drop occurred, two or more successive nights where temperatures were above 15^{o}C were necessary for renewed, significant spore production and release (Berger, 1969). These findings resulted in significant cost savings to the celery industry, as commercial growers were able to omit fungicide applications during times when few spores were likely to be dispersed.

New aspects of epidemiology are becoming apparent which may increase our understanding of dispersal mechanisms of many diseases, including species of *Cercospora*. When conditions are unfavourable for vegetative growth, many fungal species may use microcycle conidiation, the production of spores following conidial germination without an intervening phase of vegetative growth, as a dispersal mechanism. Lapaire & Dunkle (2003) hypothesised that primary conidia of *C. zeae-maydis* may be dispersed from maize residue to soybean or weed species and that after a cycle of microcycle conidiation, secondary conidia may be dispersed to other plants or fields of maize. Because this process requires less than 48 hours to complete, they suggested that this form of inoculum may be significant in disease spread and progress, and that relative humidity and intermittent periods of wetness could play a significant role in this process. For example, when secondary conidia were exposed to unfavourable conditions, such as periods of low humidity, they would dehydrate and collapse, and could be liberated by wind speeds approximately one-third of those required to liberate

hydrated conidia. Furthermore, the authors stated that the secondary conidia could rehydrate and germinate normally following dispersal (Lapaire & Dunkle, 2003).

Epidemiological information such as this is required to gain an understanding of dispersal mechanisms employed by *C. zonata* and the factors that affect spread and severity of cercorspora leaf spot. This knowledge could be used to identify climatic factors that may have influenced the increased prevalence of the disease in southern Australia.

2.6 HOST RESISTANCE

At the beginning of this project disease resistance was the highest priority of the faba bean breeding program in southern Australia, followed by quality and yield (pers com., Jeffrey Paull, 2005). Genetic resistance is the preferred choice for managing disease, since this eliminates the need to apply expensive protective fungicides and is typically effective irrespective of seasonal conditions. Currently, the national breeding program for faba beans target resistance, or decreased susceptibility, to ascochyta blight, chocolate spot, rust and viruses. Knowledge on CLS is limited and the assessment of resistance to this disease within faba bean germplasm has not been reported. In addition, rating this disease in the field is difficult as symptoms are easily confused with those of ascochyta, chocolate spot or alternaria leaf spot, especially late in the season. Nevertheless, genetic diversity may exist within the Australian germplasm and resistance to this disease could be explored. Host resistance plays an important part in disease development or control, which has been evident in other hosts where related species of C*ercospora* are recognised as causing constraints to production. Two case studies will be briefly examined to emphasise different aspects of this relationship.

Leaf spot of sugar beet was not recognised as economically important to the sugar beet crop produced in the Red River Valley and southern Minnesota region of the United States of America until 1980, when widespread epidemics of CLS were experienced. This was

attributed to the adoption of highly susceptible cultivars by the industry, allowing disease establishment and spread during weather favourable for CLS in 1979/80 (Windels et al., 1998). Following the epidemics, the industry adopted stringent selection policies for approval of new cultivars, only releasing cultivars with reduced susceptibility. Furthermore, the industry imposed a moratorium on the planting of susceptible cultivars in 1982 in an attempt to reduce the risk of repeat epidemics (Windels et al., 1998). These actions were supported in subsequent studies on the disease, which showed that cultivar resistance substantially influences the onset and progression of an epidemic of CLS in sugar beet and that epidemic onset could be delayed by 2-4 weeks in cultivars with low susceptibility (Wolf & Verreet, 2002). However, since four or five genes are responsible for this resistance, combining high levels of resistance in cultivars of sugar beet while maintaining yield is difficult (Smith & Cambell, 1996). Therefore, commercial cultivars generally have only moderate levels of resistance and require fungicide applications to obtain adequate protection (Miller et al., 1994; Khan & Smith, 2005).

Disease caused by *C. zebrina* Pass. can cause substantial losses in herbage and seed yields of highly susceptible subterranean clover (Barbetti, 1991). Cercospora disease has been reported on clovers in Western Australia (Barbetti 1985), eastern Australia (Valder, 1954) and in North America (Hanson, 1953; Berger & Hanson, 1963b) and the pathogen appears to be well adapted to the Mediterranean-type environment of south-west Western Australia (Barbetti & Nichols, 2005). Barbetti & Nichols (1994) recommended that breeding material and new cultivars in the pasture breeding programs be screened for cercospora resistance as a precautionary management strategy to protect the 6.5 million ha industry. Subsequently, Barbetti & Nichols (2005) reported on field screening trials of 96 genotypes of *Trifolium subterraneum* var. *subterraneum* and var. y*anninicum* that showed over 50% exhibited resistance to *C. zebrina*. Furthermore, the authors concluded that there was circumstantial evidence of differences for cercospora resistance among ecotypes collected

from different regions around the world. This highlighted that sources of resistance could be identified in material from overseas collections and incorporated into Australian germplasm.

2.7 DISEASE MANAGEMENT

2.7.1 Cultural practices

Methods to control *Cercospora* species in other crops frequently include practices that manage disease spread from infested residues and the use of non-host crops in rotation. For example, *C. beticola* survives in infected sugar beet leaves and, to reduce disease severity, new plantings of beets should be at least 100 m from beet residues remaining from the previous year and grown on a minimum rotation of every third year with non-host crops (Windels et al., 1998). However, in the case of grey leaf spot in maize, survival of *C. zeae-maydis* on residue is critical (Jenco & Nutter, 1992) and tillage systems which leave as little as 10% residue cover on the soil surface may result in high disease levels in crops planted in close proximity when conditions are favourable to the disease development (de Nazareno et al., 1993). Therefore, control of grey leaf spot via tillage practice is more difficult in areas of intensive corn production, since fields containing infested debris are often close to new plantings.

Management practices that control CLS in faba beans are unclear and specific recommendations for control of the disease in southern Australia have not been formalised. The National Institute of Agricultural Botany in the United Kingdom recommended to growers that wide rotations and good crop hygiene probably offer the best means of control of CLS in faba bean crops (Thomas & Sweet, 1990). Surveys conducted by researchers at the Rothamsted Research station in the United Kingdom showed that when beans were grown three times in 5 years, there was a 25% risk of damage by soil-borne pests and diseases, but this decreased to 1% if only one crop was grown every 5 years (Dyke & Prew, 1983). The current recommendation for Australian farmers is to maintain approximately 4 years between

faba beans crops within one field, as a method to control inoculum of other major stubble-borne pathogens of faba bean (*A. fabae, B. fabae*) (Wallen & Galway, 1977; Dyke & Prew, 1983). However, anecdotal evidence that shows an increase in occurrence of CLS in southern Australia indicates that research is required to develop management strategies to control the CLS.

A relatively recent change in the management of stubble is the adoption of minimal tillage, which has led to increased retention of the previous year's faba bean crop residue on the soil surface. The beneficial effects of retaining crop residue on the soil surface, namely, buffering topsoil temperature, conserving soil moisture, reducing wind and water erosion, are often offset by the negative effects which include loss as a nutrient source in the soil profile, and providing a shelter for survival, growth and reproduction of plant pathogens (Summer et al., 1981; Boosalis et al., 1986). Therefore, this cultural practice has the potential to influence the survival and distribution of *C. zonata* and emphasises a need for further information on the effects of minimal tillage on the survival and prevalence of the disease.

2.7.2 Chemical control

In most Australian farming systems where faba beans are grown it is accepted that fungicides are required to minimise the impact of foliar pathogens. However, fungicide applications are costly and require an understanding of the effect of the pathogen on plant growth for development of more efficient strategies for control. Effective fungicides have been identified for control of ascochyta blight, chocolate spot and rust, with cultivar resistance and environmental influences dictating application frequencies. Additionally, the impact of these diseases on yield and quality is well understood. In contrast, fungicide efficacy for control of CLS in faba beans and the impact of disease control on yield are not understood. This must be addressed before recommendations for fungicide use can be made or economically justified. This has not been reported in the literature nor has it been

established informally by the industry. Some case studies of chemical control of other *Cercospora* species are examined briefly to identify a research focus for this study.

Sugar beet growers in Minnesota and North Dakota, USA rely heavily on chemical control of CLS. The industry funded research to identify new fungicides that will provide effective control of CLS (Windels et al., 1998). Many fungicides have been identified and used effectively for control of the disease in the last few decades. However, resistance of *C. beticola* to the benzimidazole class of fungicides by 1981 and, later, to fentin hydroxide by 1994, led to widespread failures in disease control. By 1998, mancozeb, thiophanate methyl, azoxystrobin, copper, and mixtures of fentin hydroxide with mancozeb or thiophanate methyl also provided inadequate control of CLS (Khan & Smith, 2005). This example suggests that although fungicides may be effective for control of *C. zonata* in faba beans, there are dangers in relying on a single fungicide or chemical-group for control.

At present, cercospora disease of subterranean clover occurs only in a few areas in Western Australia but the potential to affect high value seed crops has led to the evaluation of fungicides for control of the disease. Barbetti (1987) reported that benomyl and carbendazim provided good disease control in field trials and that only one or two applications resulted in seed yield increases up to 68%. His study also showed biteranol, chlorothalonil, propiconazole and thiophanate methyl exhibited efficacy against the disease. Any recommendations for fungicide applications in faba bean to control CLS require an understanding of efficacy against the pathogen and the yield penalty in the absence of control. The most immediate benefit to the integrated management of CLS on faba bean will be the identification of effective fungicides and the development of economical strategies to control the disease in commercial crops in southern Australia.

2.8 SUMMARY

CLS is found on faba beans in many countries throughout the world. Though it normally poses only a sporadic problem in most of these countries, it has been a steadily emerging disease in Australia since 2004, with increases in both prevalence and severity since then. Control measures have not been identified and little is known about the epidemiology, survival or spread of *C. zonata* in Australian conditions. Research on other species of *Cercospora* has indicated that they can cause serious disease on other crops and many result in significant loss of yield. In the case of CLS of sugar beet, the disease was not regarded as an important threat to the industry until widespread epidemics occurred. The development of an epidemic of CLS in faba beans is believed to be promoted by warm, wet conditions, however, this has yet to be investigated and this suggestion does not begin to explain the emergence of this disease during the cold wet months of winter. The aims of this research were to improve the understanding of the pathogen and the influence of host resistance and environment, and to investigate the prevalence and epidemiology of CLS in order to determine its impact on faba bean production in Australia.

Chapter 3.

General materials and methods

The materials and methods used in all experiments are described in brief in the relevant chapter(s). Additional detail is reported here to assist in any future studies on this pathogen and the disease.

3.1 PLANT GROWTH AND MAINTENANCE

3.1.1 Faba bean cultivars

Faba bean (*Vicia faba*) cultivars or breeding lines used in experiments (Table 3.1) were supplied by the University of Adelaide's Australian Faba Bean Breeding Program (AFBBP), located at the Waite Campus, Urrbrae, South Australia. Cultivar Farah was routinely used in experiments and was chosen based on; the availability of clean seed, a cultivar commonly adopted by growers in southern Australia, susceptibility to cercospora leaf spot (CLS) in preliminary investigations, and resistance to ascochyta blight, the symptoms of which are similar to CLS. In addition, an advanced breeding line, 1322/2, was used in some studies, as preliminary investigations indicated it was resistant to CLS and resistant to ascochyta blight, which would avoid confusion in distinguishing symptoms of these diseases. Pure seed sources of both genotypes were maintained in seed-stores at 3^{o}C by the AFBBP. Research that describes the reactions to CLS in commercial cultivars and the identification of resistance and genetics in faba bean germplasm to CLS are reported separately (Kimber & Paull, 2011).

Table 3.1: Faba bean cultivars used in experiments and their origin.

Cultivar/Line	AFBBP Pedigree	Collection Import*
Farah	Selection from cv. Fiesta	ICARDA BPL1196 B8817
1322/2	Not applicable	ICARDA L82003 B8833

* Germplasm import origin from which genotype was derived

3.1.2 Potting soil

The potting soil used in all glasshouse and growth room experiments was a University of California potting mix (Baker, 1957) produced by Plant Growth Services, Plant Research Centre, Waite Campus. The mix was prepared according to the following protocol; 1,200 L of Golden Grove washed sand was steam sterilised at 100^{o}C for 30 min. After cooling, 750 L of Teem® 100% natural sphagnum peatmoss was incorporated by mixing for 5 min using an industrial spiral mixer. After 20 min, or when the mix has cooled to below 60^{o}C, the following nutrients were added, followed by 5 min mixing:

Calcium hydroxide (hydrated lime)	1000 g
Calcium carbonate (agricultural lime)	1800 g
Slow release complete Nitrophoska (N:P:K, 12:5:14)	2000 g

The mix, which produced approximately 1800 L of potting soil, had a pH of approx. 6.3. Soil was stored in plastic bins in an enclosed, cool area prior to use. This soil was chosen for its uniformity and reproducibility and also its good drainage, to reduce the incidence of black fly larvae, which might damage developing plants.

3.1.3 Controlled environment conditions

Glasshouse cubicles or controlled environment rooms (CER) were used routinely in experiments. Each glasshouse cubicle was 3.3 x 2.5 x 2.8 m, constructed with 4 mm side-glass and 4 mm roof-acrylic, and maintained at 15-25^{o}C using evaporative cooling in near natural daylight conditions (*ca* 10% UV filtered out). Alternatively, plants were maintained in a CER with 12-h photoperiod using eight halide-incandescent lights (400W, Osram) at set temperature(s) described in each study. In both facilities, potted plants were placed on benches and watered daily with tap water using a hand-held Dramm® watering wand.

3.1.3.1 Maintenance of high humidity

Experiments conducted in the CER utilised closed plastic tents (160 × 80 × 80 cm), each fitted with an ultrasonic humidifier (KT-100A, Humidaire) using RO water, placed on benches to maintain 100% humidity, when required. During the light periods in the CER the temperature inside the closed plastic tents was generally 3–5^{o}C higher than the set temperature. When required in each glasshouse cubicle, high humidity was maintained by overhead ultra-fine misters controlled by automated solenoids, which allowed misting for 30 s every 2 h.

3.2 FUNGI

Details of isolates of *C. zonata* used in this study, including the geographical location and year in which isolates were collected, are presented in Appendix 1. Isolates used in experiments to inoculate faba bean plants are described in each experiment. A few isolates of *C. zonata* had been collected prior to this period (2000-2003) and were accessed from cultures maintained in sterile reverse osmosis (RO) water at 4^{o}C by the pulse pathology laboratory staff at the South Australian Research and Development Institute (SARDI). Identification of *C. zonata* was confirmed by; disease expression on infected plant material, description of morphology of conidia (Williams, 1987) collected from lesions on infected plant material, and growth characteristics in culture (PDA, Oxoid Ltd). Methods for collection and storage of *C. zonata* isolates are detailed in section 3.2.1.

3.2.1 Collection and storage of *Cercospora zonata* isolates

3.2.1.1 Culture media

Potato dextrose agar (PDA) was used routinely as the medium for culturing isolates of *C. zonata.* A series of experiments was conducted to examine the effect of different combinations of media, incubation conditions and techniques on growth and sporulation of *C.*

zonata, and is presented in section 4.2.1. The ingredients and preparation of all media are described in Appendix 2.

3.2.1.2 Establishment of isolates from mycelia

Leaf and stem samples were collected from infected plants in commercial faba bean crops or research trials. Unique details such as geographical location, date of collection and collector were recorded and the sample assigned an accession number (specimen number/year of collection). Standard culturing techniques used routinely to isolate a pathogen from surface sterilised portions of infected tissue proved inconsistent for routine culturing of *C. zonata*. Therefore, conidia excised directly from sporulating lesions, using aseptic techniques, were used to establish cultures of *C. zonata* as follows.

Infected plant material with distinct CLS lesions was placed in a plastic bag containing moist paper towel, sealed, then incubated at *ca* 4°C in the dark for 1-5 days. This induced sporulation of the fungus, which was generally observed near the centre of lesions. Conidia were excised from lesions using a sterilised needle and transferred to PDA (in a 9-cm diameter Petri dish). Cultures were maintained at room temperature (*ca* 22^{o}C) for 1-2 weeks under 12 h light/dark cool-white (Phillips 18W/850) and black-light (NUV) (NEC 20W/BLT10) fluorescent light. Using aseptic technique, pure cultures were derived from single hyphal tips excised from the margin of actively growing cultures, under a Leica MS5 dissecting microscope at 25x magnification, and transferred to PDA (in a 9-cm Petri dish). Cultures were maintained for 2 weeks as described above. All *C. zonata* isolates were stored as mycelium on six 3 mm 'plugs', cut using a brass cork-borer from the active growing margin of a 2-week old culture, in a 1.5 ml Nunc® cryotube containing 1 ml sterile RO water. Five and nine replicate cryotubes were prepared for each isolate, each labelled with the appropriate accession number, then stored at 4^{o}C. To retrieve an isolate from storage, two

plugs were transferred to PDA and incubated at *ca* 22°C for 2 weeks under cool-white and black fluorescent light with 12 h photoperiod as described above.

3.2.1.3 Preparation of C. zonata *inoculum – mycelia suspension*

Mass inoculum was prepared from a single culture using the following aseptic techniques. A portion of the growing culture was macerated using a scalpel (no. 22) and transferred to a 10-ml vial containing 3 ml RO water. A sterilised glass rod was used to pulverise culture pieces before the suspension was mixed using a vortex touch mixer. A 150-µl aliquot of the resulting mycelial suspension was transferred to PDA and spread with a bent-glass rod. This process was repeated for each isolate. Cultures were incubated at 22°C for 10–15 days under 12 h light/dark cool-white and black-light (NUV) fluorescent light, as described above, then combined into a commercial blender (32BL80, Waring Commercial) containing RO water to macerate into a mycelial suspension. The suspension was strained through two layers of sterile muslin cloth, and the concentration of mycelial fragments was estimated using a haemocytometer. The final inoculum concentration was adjusted by dilution with RO water and surfactant (0.05% Tween 20; BDH Laboratory Supplies), then applied to seedlings using a hand-held sprayer (Hardi C5, Hardi Australia) until run-off.

3.3 STATISTICAL ANALYSIS

Specific methods of analysis are described in each chapter; results from most experiments were analysed using ANOVA, performed in Genstat® version 11, and data were transformed to normalise distributions when required. Original data are presented in figures for clarity, generated using Excel 2003 for Windows® or GraphPad Prism® version 5.

Chapter 4.

Preliminary studies on *Cercospora zonata*

PRELIMINARY STUDIES ON *CERCOSPORA ZONATA*

4.1 INTRODUCTION

There are limited reports of research on *Cercospora zonata* of faba bean, the pathogen has not been studied intensively (see section 2.4), and those documented do not describe methodology in detail. Culturing of *Cercospora* spp. *in vitro* is difficult and methods for mass production of viable conidia on artificial media have not yet been identified for most Cercosporoid fungi (Booker et al., 2008). *C. zonata* has been isolated and cultured on PDA but fails to sporulate on this medium. Therefore, one objective of the experiments described in this chapter was to identify techniques to induce sporulation of the pathogen *in vitro* to facilitate identification of *C. zonata* and mass production of inoculum for subsequent studies.

C. zonata is thought to survive between crop seasons as conidiophores or dormant mycelium on infested faba bean debris (Yu, 1947; Walker, 1952) and transmitted by air-borne, or rain-splash dispersed, conidia (Williams, 1987). Survival on crop debris is an important source of inoculum for many species of *Cercospora* (Payne & Waldron, 1983; Windels et al., 1998). Those pathogens which reside within residue often decline rapidly when host residues are buried, a result of the rapid decay of host material in the soil profile (Wallen & Galway, 1977; Dyke & Prew, 1983). However, the high incidence of CLS observed in research trials and commercial crops of faba bean in southern Australia in recent seasons often occurred despite 3-4 year crop rotation practices, suggesting that inoculum may survive for long periods in the soil profile. Therefore, the second objective of the preliminary studies described here was to examine the mechanisms of survival of *C. zonata* and the relative importance of stubble-borne *versus* soil-borne inoculum using a bioassay technique. The outcome of these experiments aimed to contribute further information on the viability of CLS inoculum within a soil profile.

4.2 MATERIALS AND METHODS

4.2.1 *In vitro* sporulation of *Cercospora zonata* on artificial media

The following media were reported to induce sporulation in some species of *Cercospora* (Dhingra & Sinclair, 1995) and were selected for evaluation of *C. zonata*; Bean Seed Agar (BSA), Carrot Juice Agar (CJA), Carrot Leaf Decoction Agar (CLDA), Cornmeal Agar (CMA) and V8 juice Agar (V8A). Other media included were modifications of these recipes; Carrot Juice Agar (CJA2), in which unfiltered purée of carrots was used instead of filtered juice, Carrot Leaf Pulp Agar (CLPA), in which unfiltered carrot leaf purée was used instead of filtered juice, and V8 juice Broth (V8B). The ingredients and preparation of each medium are described in Appendix 2. A series of experiments was conducted to examine the effect of different media combined with incubation conditions and culturing techniques on growth and sporulation of *C. zonata*.

4.2.1.1 Effect of medium, light and culturing on mycelial growth and sporulation of C. zonata

Six media were examined; CJA, CJA2, CLDA, CLPA, V8A and BSA, with two lighting regimes; continuous white and near ultraviolet (NUV) fluorescent light or 12 h light/dark. *C. zonata* isolate 36/04 (Appendix 1) was cultured using two different aseptic techniques to inoculate media plates; (i) a 3 mm 'plug' from the active growing margin of a 2-week old *C. zonata* culture, cut using a sterile brass cork-borer, was placed in the centre of each plate, and (ii) a 200 µl aliquot of mycelial suspension (prepared as described in section 3.2.1.3) was added to each Petri dish immediately following pouring and distributed through the medium using a wire loop. The plate was then gently agitated and allowed to cool and set. A total of 36 plates (6 media x 2 treatments x 3 replicates) was incubated at room temperature (approximately 22^{o}C) under each lighting regime. The cultures were inspected for evidence of sporulation every 3 days under a binocular microscope (x20) and putative spore

development examined using a compound microscope (x100). The diameter of colonies that developed from mycelial plugs was measured along two perpendicular transects on days 14, 35 and 56. The mean of the two transects was calculated to examine growth rate over time and the standard error (SE) calculated for each treatment. Detailed statistical analysis was not performed when sporulation was not apparent. The colony growth of the mycelial suspension method was measured once, at 56 days after inoculation, recorded as the percentage of the Petri dish covered by mycelium.

4.2.1.2 Effect of culture maintenance on sporulation

A series of laboratory experiments was conducted in which media, culturing technique and incubation conditions were varied to identify techniques to induce sporulation of *C. zonata* in culture. The methodology of each experiment, including the number of *C. zonata* isolates (replicates), varied. Standard techniques for culturing *C. zonata* are presented in section 3.2.1. Brief descriptions of the methods examined in this study are presented in Table 4.1 and details for the preparation of media are presented in Appendix 2. The cultures were inspected for signs of sporulation every 3 days under a binocular microscope (x20) and putative spore development examined using a compound microscope (x100).

Table 4.1: Outline of preliminary experiments (shaded blocks) to evaluate culturing techniques and incubation conditions to induce isolates of *Cercospora zonata* to sporulate *in vitro* using different types of artificial media, as described in section 4.2.1. Tick (✓) indicates medium was included in the experiment.

***C. zonata* isolates**	**Variations in methodology examined**	**BSA**	**CJA**	**CJA2**	**V8A**	**CLDA**	**CLPA**	**V8B**	**CMA**
FT04024 36/04 37/04 71/04 29/04	Two light regimes; cycle of 12 h day/night white & NUV fluorescent or 7 days of complete darkness followed by 12 h day/night white & NUV fluorescent. 250 µl aliquots of mycelial suspension were mixed into medium or into broth (V8B). Examined after 21 days.	✓			✓			✓ 45%	
36/04 69/04	Three concentrations of V8 broth. Incubated in complete darkness. 3 ml aliquots of mycelia suspension were incorporated into 80 ml broth before pouring. Examined after 21 days.							✓ 20% 50% 90%	
36/04	Two replicates of *C. zonata* isolate 36/04 were included. 2-week old cultures were incubated at 4°C under 12 h day/night white fluorescent light and examined after 14 days.	✓	✓	✓	✓	✓	✓		
36/04 69/04	Thick V8A, by pouring 40ml molten V8 agar into 9 cm petri dish. 3 mm culture plug placed onto medium. Two light schedules; continuous or 12 h day/night white & NUV fluorescent. Examined after 21 days.				✓				
37/04 73/04 74/04	3mm culture plug placed onto medium and incubated at 23°C under 12 h light/dark white & triphosphor fluorescent light, until 1/3rd coverage of medium, then transferred to room temperature under 12 h day/night NUV fluorescent light. Examined after 21 days.								✓

Note: Isolates were cultured using techniques described in section 3.2.1 and incubated at *ca* 22°C, unless otherwise stated. Concentrations of V8 juice Broth (V8B) evaluated are represented by a percentage (%). Media used were; Bean Seed Agar (BSA), Carrot Juice Agar (CJA), unfiltered Carrot Juice Agar (CJA2), V8 juice Agar (V8A), Carrot Leaf Decoction Agar (CLDA), Carrot Leaf Pulp Agar (CLPA) and Cornmeal Agar (CMA). Each *C. zonata* isolate represents a replicate in each experiment.

4.2.2 Stubble- and soil-borne inoculum

A series of experiments was conducted in a CER, maintained at 15/10^{o}C day/night. The CER was fitted with high humidity chambers, as described in section 3.1.3.1. The role of soil and faba bean residue in survival of *C. zonata* was examined, using soil and infested residue samples collected after harvest in December 2005 from a field research site where cercospora leaf spot had been severe on faba bean in untreated field plots that season. Soil samples were stored dry at 4^{o}C until required and residue samples were stored dry at room temperature until prepared as described for each experiment. The experiments were undertaken to address two aims:

a) To develop a plant bioassay that measured the 'infectivity' of infested stubble and soil on faba bean seedlings.

b) Provide preliminary information on pathogen survival and dispersal.

Preliminary experiments (not presented in detail) were conducted by placing infested soil or residue (milled or threshed) on the surface of University of California (UC) potting mix sown with seeds of faba bean cv. Farah. These pots were maintained in the CER and watered routinely overhead. Seedlings remained exposed to the infested residue or soil for the duration of the experiment and monitored for disease over 4 weeks. Few lesions typical of CLS were observed on leaves of faba bean seedlings in these preliminary experiments. Therefore, in subsequent experiments, alternative methods for the bioassay were examined and are presented in more detail below.

4.2.2.1 Soil bioassay

Four inoculation treatments were evaluated to estimate the infectivity of soil infested with *C. zonata* 4 months after it was collected from the field (Table 4.2). The experiment was arranged as a randomised complete block design (RCBD) with four replications. The faba bean plants (cv. Farah) were maintained in high humidity chambers fitted with ultrasonic humidifiers.

Disease was assessed 4 weeks after the final inoculation treatment (Treatment 4), when the number of CLS lesions on each seedling was counted. Detailed statistical analyses were not conducted, as this was a preliminary experiment to determine methodology, thus data were compared using means and SE of treatments.

Table 4.2: Treatments designed to estimate the infectivity of field soil samples infested with *Cercospora zonata* when inoculated onto faba bean seedlings (cv. Farah) in a bioassay maintained in high humidity, using an ultrasonic humidifier, at 15/10°C day/night in a controlled environment room (CER). Plants were assessed for disease 4 weeks after inoculation as number of lesions per seedling.

Treat 1	Soil sub-sample (60 g) was sprinkled evenly over the surface of the soil prior to seedling emergence then maintained in high humidity. Pots were watered daily.
Treat 2	Soil sub-sample (60 g) was sprinkled evenly into a wire basket (0.8 mm pores) suspended 6 cm above the soil in each pot prior to emergence and watered through the basket daily, then removed 4 days after plant emergence.
Treat 3	Soil sub-sample (60 g) was sprinkled evenly into a wire basket (0.8 mm pores) containing a paper-filter cup (Thomas & Green, Autocup®) and suspended 6 cm above the soil surface in each pot prior to seedling emergence and watered through the basket daily, then removed 4 days after plant emergence. See Figure 4.1A
Treat 4	Soil samples (each 240 g) were each flooded in 400 ml RO water at *ca* 22°C for 4 h, mixed frequently, then allowed to settle for 60 min, after which the 300 ml of supernatant was decanted through a coarse sieve to remove floating debris. The suspension (plus 0.2% Tween-20 surfactant) was applied to 4-week old seedlings using a hand sprayer until runoff.

Note: Each pot (150 mm diam) was filled with UC potting mix and sown with four seeds of cv. Farah at a sowing depth of 3 cm before treatments were applied. Four replicates were included for each treatment.

4.2.2.2 Stubble bioassay

Four inoculation treatments were evaluated to estimate the infectivity of infested faba bean residues (cv. Farah) 4 months after they were collected from the field (Table 4.3). The residues were threshed (fragmented) to produce pieces *ca* 10mm in length. A 40 g sub-sample was used in each treatment, applied to emerging faba bean seedlings (cv. Farah) planted in UC potting mix. The experiment was arranged as an RCBD with three replications and treated plants were maintained in high humidity chambers fitted with ultrasonic humidifiers within the CER. Disease was assessed 4 weeks after the final inoculation treatment (Treatment 2), when

the number of CLS lesions on each seedling was counted. Detailed statistical analyses were not conducted as this was a preliminary experiment to determine methodology, thus data were compared using means and SE of treatments

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Table 4.3: Treatments used to estimate the infectivity of faba bean residue infested with *Cercospora zonata* on faba bean seedlings (cv. Farah). Treatments were applied to plants and maintained in high humidity at 15/10^{o}C day/night within a controlled environment room (CER). Plants were assessed for disease 4 weeks after inoculation.

Treat 1	Residue sub-sample (40 g per pot) was sprinkled evenly over the surface of potting soil prior to seedling emergence, watered and covered with moistened cloth until seedling emergence.
Treat 2	Residue sub-sample (160 g) was flooded in 1 L RO water for 24 h then the suspension decanted through a 15 µm sieve. The sieved fraction (>15 µm) was resuspended in 200 ml RO water and inoculated onto 2-week old faba bean seedlings using a hand sprayer (plus 0.2% Tween-20 surfactant) until runoff.
Treat 3	Prior to seedling emergence, a residue sub-sample (40 g per pot) was sprinkled evenly into a wire basket (0.8 mm pores) suspended 6 cm above the soil surface in each pot and saturated with RO water, then covered with moistened cloth and maintained above the pot for 4 days after seedling emergence.
Treat 4	Prior to seedling emergence, a residue sub-sample (40 g per pot) was sprinkled evenly into a wire basket (0.8 mm pores) suspended 6 cm above the soil surface in each pot and saturated with RO water, then left uncovered and maintained above the pot for 4 days after seedling emergence.

Note: Each pot (150 mm diam) was filled with UC potting mix and sown with four seeds at 3 cm sowing depth before treatments were applied. Four replicates were included for each treatment.

4.2.2.3 Residue and soil bioassay

The most effective treatments identified in the previous bioassay experiments (Treatment 3 in Table 4.2; Treatment 2 in Table 4.3) were selected to examine the infectivity of *C. zonata*-infested faba bean residue and soil 12 months after they were collected from the field. Infested residues were milled (Wiley® Hammer Mill) to produce finely fragmented pieces (*ca* 4mm) and aggregations within soil samples were shattered by hand. The experiment was arranged as a RCBD with four replications and was conducted in a CER, with high humidity maintained after treatments were applied. Forty-eight pots (150 mm diameter) were filled with UC potting mix and each sown with four seeds of cv. Farah, thinned to three seedlings after emergence. Five

treatments were examined (Table 4.4) and an un-treated control was included. Four treatments (Treat 2 to 5) were prepared as suspensions, with surfactant (0.05% Tween-20), and derived consecutively from infested residue and soil samples (Table 4.4). Suspensions were applied to 4-week old seedlings until run-off using a hand-held spray applicator. After treatments were applied, plants were maintained in high humidity for 6 days, after which the humidifiers were switched off and tents opened to promote air-flow. Seedlings were assessed for disease 21 days after the final treatment (Treatment 5) to allow adequate time for symptoms to be expressed in all treatments, by counting the number of CLS lesions on leaves, and recording defoliation as the number of dropped leaves. Results were analysed using a general linear model - ANOVA, performed in Genstat® version 11.

Table 4.4: Treatments used to estimate the infectivity of faba bean residue and field soil infested with *Cercospora zonata* on faba bean seedlings (cv. Farah). Treatments were applied to plants as described then maintained in high-humidity for 6 days after inoculation within a controlled environment room (CER) at 15/10°C day/night. Plants were assessed for disease 21 days after inoculation.

Treat 1	Residue (30 g) or soil (50 g) sample was sprinkled evenly into a wire basket, containing a paper-filter cup (Thomas & Green, Autocup®) and plastic collar to retain sample, suspended 6 cm above the soil surface in each pot, prior to seedling emergence and watered through the basket daily, then removed 5 days after seedling emergence. See Figure 4.1.
Treat 2	Residue (160 g) or soil (200 g) sample was flooded in 1600 ml and 400 ml RO water, respectively and incubated at 15°C for 3 h then the suspension was decanted, sieved through a 430 µm filter and inoculated onto four-week old seedlings.
Treat 3	Residue or soil sample retained from Treat 2, which had been incubated moist for 1 day at 15°C, was flooded in 400 ml of RO water for 30 min, then the suspension was decanted, sieved through a 430 µm filter and inoculated onto four-week old seedlings.
Treat 4	Residue or soil sample retained from Treat 3 after 3 days moist-incubation at 15°C, was flooded in 400 ml of RO water for 30 min, then the suspension was decanted, sieved through a 430 µm filter and inoculated onto four-week old seedlings.
Treat 5	Residue or soil sample retained from Treat 4 after 5 days moist-incubation at 15°C, was flooded in 400 ml of RO water for 30 min, then the suspension was decanted, sieved through a 430 µm filter and inoculated onto four-week old seedlings.

Note: Each pot was filled with UC potting mix and sown with four seeds at 4 cm sowing depth before treatments were applied. Four replicates were included for each treatment.

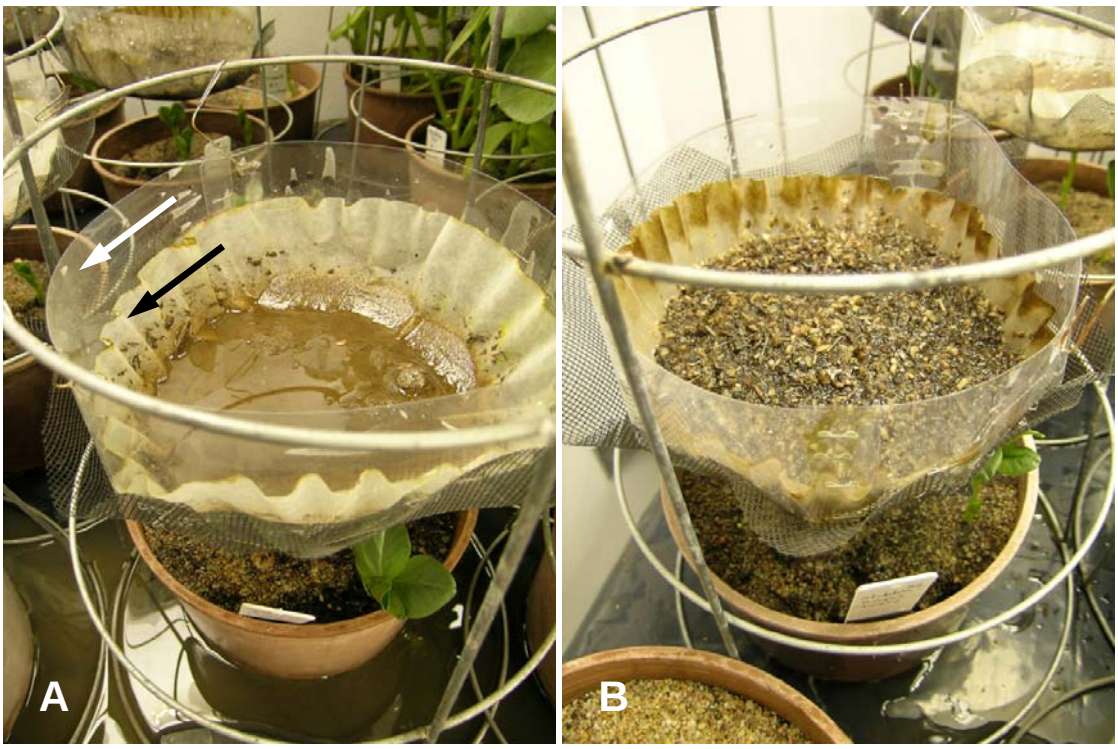

Figure 4.1: Inoculation technique (Treat 1) used in a bioassay conducted in a controlled environment room (CER) to examine infectivity of faba bean residue and field soil infested with *Cercospora zonata* on faba bean seedlings (cv. Farah). A wire basket (0.8 mm pores) supported a paper-filter cup (Thomas & Green Autocup®) (black arrow) and a plastic retaining collar (white arrow) which contained 30 g soil samples (**A**), or 50 g milled faba bean residue sample (**B**), suspended 6 cm above the soil surface in each pot prior to emergence. Treatments were maintained in high humidity within the CER, and watered daily through the basket, then removed 6 days after seedling emergence.

4.2.3 Infectivity of *C. zonata* in soil fractions

The survival of *C. zonata* in soil was further examined, by means of collecting filtrates from soil to inoculate onto plants, as determined in previous bioassay treatments (Treatments 2 to 5, Table 4.4). Soil samples collected from a CLS field trial in 2005, as described above, then stored for 12 months were used to determine the size of CLS propagules residing in the soil that may infect faba bean. The experiment was arranged as an RCBD with four replications. Thirty-two pots (150 mm diam.) were filled with UC potting mix and each sown with four seeds of cv. Farah, thinned to three seedlings after emergence. A 250 g sample of soil was submerged in 400 ml RO water, agitated, and incubated at room temperature (*ca* 22°C) for 3 h, agitating the mixture every 30 min. The suspension was then stirred and passed through a 430 µm filter to remove large particulates after which six 'soil filtrate' treatments were produced by passing the

soil suspension through a column of five sieves; 250, 150, 105, 50 and 15 µm. The particles captured in each sieve were collected by re-suspension in 50 ml RO water, resulting in five soil filtrate treatments. A sixth treatment of <15 µm particulates was created from a 50 ml sub-sample of the final suspension collected at the bottom of the sieve column. This process was repeated for each soil sample, treated as a replicate. Surfactant (0.05% Tween-20) was added to each soil filtrate treatment and then the suspension inoculated onto a pot of three seedlings, at the four-leaf growth stage, using a hand-held spray applicator. Control plants were sprayed with RO water plus surfactant. Immediately after inoculation, plants were placed in high humidity within the CER for 6 days. Plants were assessed for disease 28 days after inoculation, as the number of CLS lesions on leaves, and defoliation, as the number of leaves that abscised. Results were analysed using a general linear model - ANOVA, performed in Genstat® version 11.

4.3 RESULTS

4.3.1 *In vitro* sporulation of *Cercospora zonata* on artificial media

4.3.1.1 Effect of medium, light and culturing on mycelial growth and sporulation of C. zonata

Reproductive structures, such as conidia or conidiophores, of *C. zonata* were not observed in culture using any of the artificial media, culturing techniques or lighting regimes evaluated in this study. Therefore, detailed statistical analyses were not conducted as sporulation was the main objective of this study and mycelial growth rate was of minor interest. The growth of *C. zonata* in culture, measured as the mean diameter of the growing culture (mm day^{-1}), was slow but slight differences were observed between media incubated under the two lighting regimes at room temperature. The mean growth rate of *C. zonata* on all media evaluated was 1.45 mm day^{-1} when incubated under continuous lighting (24 h light) compared to 1.34 mm day^{-1} when cultures were incubated under a 12 h photoperiod. Slight variation (±0.3 mm day^{-1}) in growth rate was observed among cultures of *C. zonata* isolate 36/04 grown on six different media (Figure 4.2). The slowest growth rate was observed on V8A (1.26 mm day^{-1}) and the

fastest on CLPA (1.57 mm day^{-1}), both under continuous lighting. The pathogen showed the same growth rate trend among both media type and lighting regime for both culturing methods examined, i.e. from a mycelial plug or from mycelial suspension distributed within the medium (data not shown).

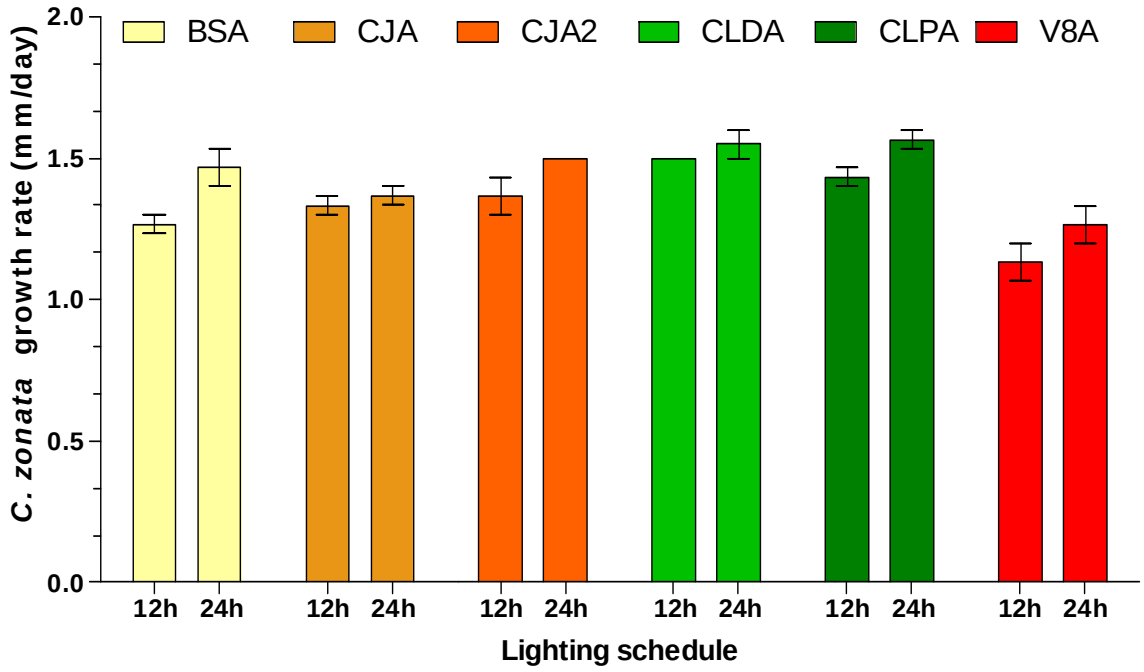

Figure 4.2: Mean growth rate (mm day^{-1}) of *Cercospora zonata* (isolate 36/04) from a 3-mm culture plug on six different artificial media incubated at room temperature (*ca* 22^{o}C) under two lighting regimes; 24 h or 12 h white and NUV fluorescent light. Bars indicate the standard error of the mean.

4.3.1.2 Effect of culture maintenance on sporulation

No conidia or conidiophores of *C. zonata* were observed in any of the conditions described in Table 4.1. Consequently detailed statistical analyses of results were not performed, as the objective of the experiments was to evaluate methods to induce sporulation of *C. zonata* in culture.

4.3.2 Stubble- and soil-borne disease inoculum

No lesions typical of CLS were observed on leaves of faba bean seedlings in the preliminary experiments when infested soil or residue (milled or threshed) was placed on the soil surface prior to seedling emergence.

4.3.2.1 Soil bioassay

Distinct leaf lesions, characteristic of CLS, were observed on faba bean seedlings 28 days after inoculation using soil collected from the 2005 CLS field trial. Most lesions (average 13.3 lesions per plant) were observed on seedlings exposed to moistened soil samples that had been suspended in a filter-paper basket above seedlings during emergence (Treat 3) (Figure 4.3). Substantially fewer lesions (average <2.6 lesions per plant) were observed on seedlings when soil samples were; applied directly to the soil surface prior to emergence (Treat 1), suspended overhead in an open-mesh wire basket during emergence (Treat 2), or soaked in water to extract a suspension that was inoculated onto seedlings (Treat 4) (Figure 4.3).

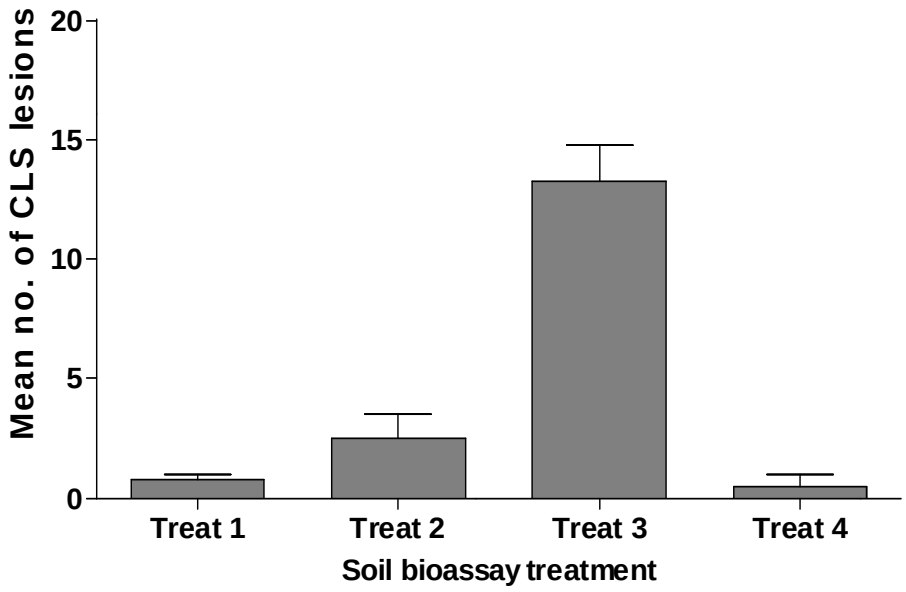

Figure 4.3: Mean number of cercospora leaf spot (CLS) lesions on faba bean seedlings (cv. Farah) inoculated with *Cercospora zonata*-infested field-soil samples in a controlled environment room (CER) bioassay maintained at 15^{o}C/12^{o}C day/night. Inoculation methods used were; **Treat 1**, sample placed on soil surface prior to emergence, **Treat 2**, sample suspended in wire basket 6 cm above the soil until 4 days after plant emergence, **Treat 3**, sample suspended in a wire basket within a paper-filter 6 cm above the soil until 4 days after plant emergence, **Treat 4**, a filtrate from soil, that had been flooded in RO water for 4 h, inoculated onto four-week old seedlings. Plants were maintained in high humidity within the CER and assessed for disease 4 weeks after inoculation with Treat 4. Bars indicate standard error of the mean.

4.3.2.2 Stubble bioassay

Distinct lesions, characteristic of CLS, were evident on leaves 28 days after inoculation using faba bean residue infested with *C. zonata*. Most lesions (average 38.5 lesions per plant) were observed on seedlings inoculated with a filtered suspension (containing particles ≤50 μm) from washed infested stubble samples that had been soaked in RO water for 24 h (Treat 2) (Figure 4.4). Few leaf lesions (< 1.0 lesion per plant) were observed on seedlings inoculated by other methods described in Table 4.3. There were no symptoms of other foliar diseases visible on seedlings in any bioassay treatment examined.

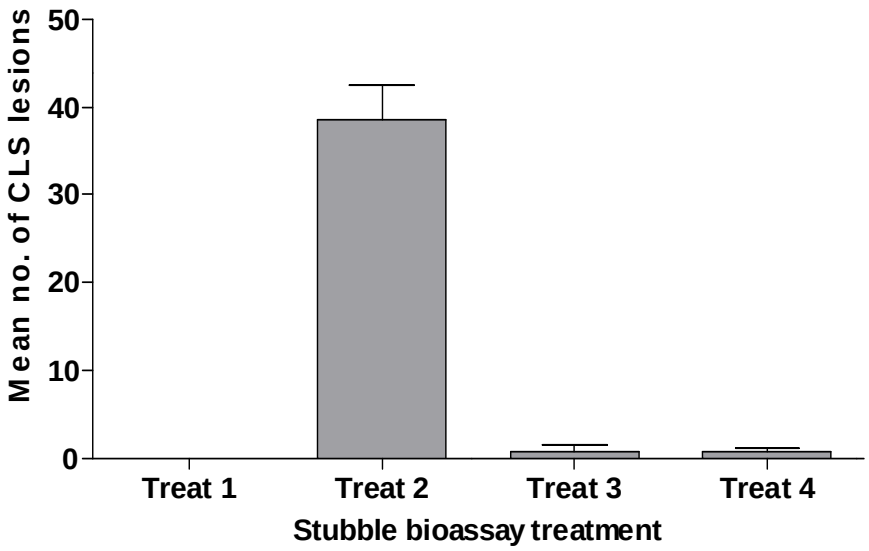

Figure 4.4: Mean number of cercospora leaf spot (CLS) lesions observed on faba bean seedlings (cv. Farah) inoculated with samples of *Cercospora zonata* infested faba bean residue (*ca* 10 mm fragments) in a controlled environment room (CER) bioassay, conducted four months after residues were collected from the field, maintained at 15°C/12°C day/night. Inoculation methods used were; **Treat 1**, residue sample was placed on the soil surface prior to plant emergence, **Treat 2**, two-week old seedlings were inoculated with a filtered suspension (≤50 μm) extracted from residue that had been flooded for 4 h in RO water, **Treat 3**, residue sample was suspended in a wire basket 6 cm above the soil, covered with wet cloth, then removed 4 days after seedling emergence, **Treat 4**, residue sample was left uncovered and suspended 6 cm above the soil surface until 4 days after seedling emergence. Plants were maintained in high humidity within the CER and assessed for disease 4 weeks after inoculation with Treat 2. Bars indicate standard error of the mean.

4.3.2.3 Residue and soil bioassay

Necrotic lesions, characteristic of infection by *C. zonata*, were evident on leaves of faba bean seedlings 21 days after being inoculated with filtrates from soil and stubble (Figure 4.7). Analysis of the mean number of CLS lesions on seedlings showed a significant interaction ($P<0.05$) between inoculum source (soil or stubble) and inoculation treatment. CLS lesions developed on leaves that were inoculated with soil filtrates collected after four different periods of saturation in water (Treatments 2-5, Table 4.4). Significantly more ($P<0.05$) lesions (9.25 lesions/plant) were observed on seedlings inoculated with the soil filtrate from the shortest incubation period (3 h) in RO water, than with soil filtrates extracted following longer saturation periods i.e. <1.5 lesions/plant after saturation periods of 1, 3 or 5 days (Figure 4.5). In comparison, very few leaf lesions (<1.5 lesions/plant) were produced following inoculation with filtrates from infested faba bean residue after short saturation periods of 3 h and 1 day (Treatments 2 and 3), and disease was not observed on seedlings inoculated with filtrates extracted from stubble following incubation for 3 and 5 days (Figure 4.5). No disease was observed in treatment 1, where soil or stubble samples were suspended over the bioassay pots during seedling emergence.

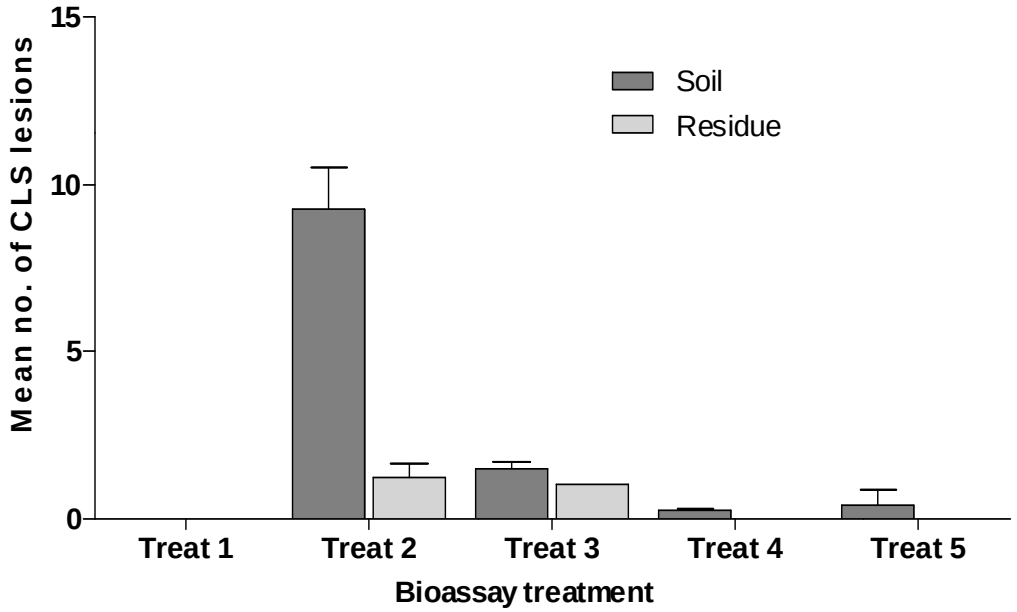

Figure 4.5: Mean number of cercospora leaf spot (CLS) lesions observed on faba bean seedlings (cv. Farah) 21 days after five inocula, prepared from samples of *Cercospora zonata*-infested faba bean residue and field-soil, were applied to plants in a controlled environment room (CER) at 15/10^{o}C day/night. The bioassay was conducted 12 months after samples were collected from the field. Inoculation treatments used were; **Treat 1**, sample was suspended 6 cm above the soil surface in a paper-filter until 5 days after seedling emergence; **Treat 2**, inoculated with filtrate from sample after flooded in 400 ml RO water for 3 h; **Treat 3**, inoculated with filtrate from sample (retained from Treat 2) after 1 day moist-incubation then flooded in RO water for 30 min; **Treat 4**, inoculated with filtrate from sample (retained from Treat 3) after 3 days moist-incubation then flooded in RO water for 30 min; and **Treat 5**, inoculated with filtrate from sample (retained from Treat 4) after 5 days moist-incubation then flooded in RO water for 30 min. Samples were incubated within the CER and plants were maintained in high humidity for 6 days after inoculation, after which humidity was removed. $LSD_{(0.05)}$ sample*treatment = 1.28. Bars indicate standard error of the mean.

4.3.3 Infectivity of *C. zonata* in soil fractions

Lesions, characteristic of *C. zonata* infection, were observed on faba bean seedlings 28 days after inoculation with the different fractions of soil filtrates. There was a significant difference ($P<0.05$) in the mean number of leaf lesions following inoculation with fractions from the infested soil (Figure 4.6). The most CLS lesions (11.4 and 8.1 lesions per seedling) were observed on seedlings inoculated with soil fractions with particle size ranges of 15-50 µm (Figure 4.7B) and 50-105 µm (Figure 4.7C), respectively. Significantly fewer ($P<0.05$) lesions (< 1.8 lesions per seedling) were observed on seedlings inoculated with soil fractions comprising

particles < 15 µm (Figure 4.7A) or greater than 105 µm (Figure 4.7D, E, F), and these were not significantly different from the control treatment (Figure 4.6). No other foliar diseases were observed on the treated plants.

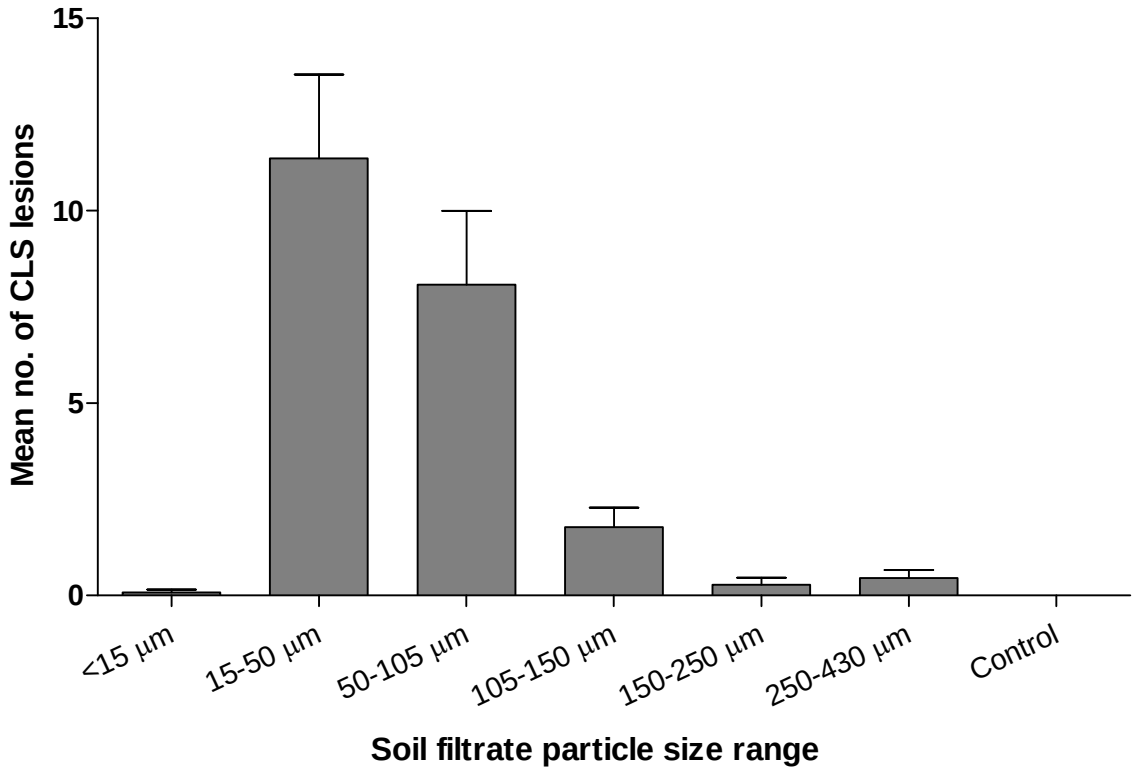

Figure 4.6: Mean number of cercospora leaf spot (CLS) lesions on faba bean seedlings (cv. Farah) 28 days after inoculation with six fractions from field-soil samples infested with *Cercospora zonata*, prepared after passing soil through a column of sieves and resuspending captured particles. Control plants were sprayed with RO water plus surfactant. Plants were incubated in a controlled environment room (CER) at (15/10^{o}C day/night). $LSD_{(0.05)}$ = 3.57. Bars represent standard error of the mean.

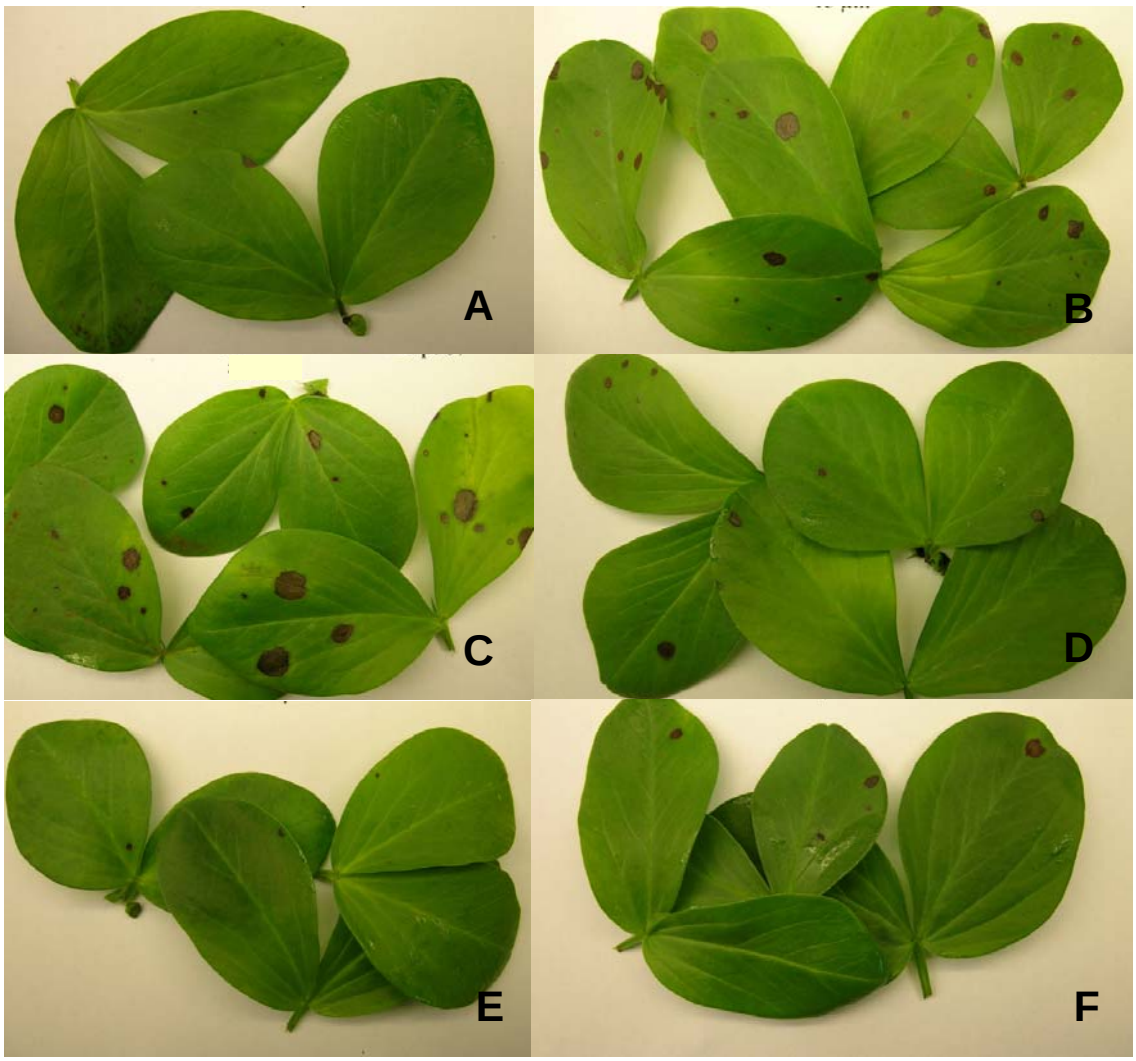

Figure 4.7: Representative cercospora leaf spot (CLS) lesions on faba bean leaves (cv. Farah) 28 days after inoculation with six different fractions from *C. zonata* infested field-soil samples in a controlled environment room (CER) bioassay (15/10^{o}C day/night). Soil samples were washed through a column of sieves and captured particles were resuspended to produce fractions of <15 µm (**A**), 15-50 µm (**B**), 50-105 µm (**C**), 105-150 µm (**D**), 150-250 µm (**E**) and 250-420 µm (**F**). Plants were maintained in high humidity for 6 days after inoculation.

4.3.4 Discussion

None of the techniques or artificial media tested in this study induced sporulation of *C. zonata* in culture. This outcome reflects similar reports of difficulties in culturing other *Cercospora* spp. and highlights the ongoing absence of methodology for mass production of conidia of Cercosporoid fungi on artificial media (Booker & Umaharan, 2008). There was slight variation in growth rate of *C. zonata in vitro* among different media evaluated in this study. However, growth rates were not substantially different compared to standard culturing techniques using PDA, described in section 3.2.1, and most were more complicated in ingredients and preparation. Therefore, PDA was chosen as the medium for culturing in subsequent experiments and mycelial suspensions were used as inoculum, as described in section 3.2.1.3.

The preliminary investigations of *C. zonata* in infested faba bean stubble and soil allowed establishment of a plant bioassay in a controlled environment. The bioassay was used to estimate the 'infectivity' of infested residue or soil collected from the field as a source of inoculum for CLS. Infection of plants by *C. zonata* from either source of inoculum differed with the inoculation technique used. The bioassay experiments showed that 4 months after samples were collected from the field, negligible transmission of CLS occurred by splash dispersal when infested soil or stubble was placed directly on the surface of the potting mix during seedling emergence. However, CLS lesions formed when samples were suspended above the seedlings during emergence, such that seedlings were exposed to water that had flowed through the samples. Disease was most severe when water had passed through samples contained within a paper filter-cup (Thomas & Green Autocup®). The porosity of these filters is not stated in the manufacturer's specifications, but it was estimated that particulates less than 150 μm would pass through the filter (pers. comm., Crown Scientific, June 2010). However, only negligible disease occurred on plants when infested faba bean residue samples were assessed using this technique 4 months after collection (section 4.3.2.2). Only when infested residue was flooded with water

and the filtrate was used to inoculate plants, were significant amounts of disease observed. This inoculation technique with flooding was also used in the first soil bioassay (4.3.2.1) on infested soil samples but yielded few leaf lesions. The discrepancy may be due to variation in the methodology, as the filtrate extracted from soaked soil samples was allowed to settle prior to decanting, which may have led to a significant portion of particulates settling out from the suspension.

The combined soil and stubble bioassay allowed estimation of infectivity 12 months after samples were collected from the field, using filtrates from four different saturation periods. Disease was most severe on plants inoculated with filtrates of soil samples that had been flooded in water for 3 h. Few lesions were observed on plants inoculated with filtrates from the soil sample after 1, 3 or 5 days of moist incubation periods. The infectivity of residue samples was significantly lower than that of soil in this same experiment. A similar trend was evident, however, as leaf lesions occurred on plants inoculated with filtrates from stubble samples flooded in water for 3 h and 1 day but not if incubation was longer. These results suggest that propagules of *C. zonata* residing in infested soil and residue are disseminated after short periods of wetness and may be exhausted rapidly. Furthermore, a 95% reduction in infectivity on faba bean from infested residue was observed over time, shown in bioassays conducted 4 and 12 months after samples were collected. Only a 30% reduction in infectivity over time was observed for infested soil samples. This may indicate that soil has a major role, compared to infested residue, in harbouring primary inoculum of *C. zonata* over time. However, the effect of storage was not examined in this study, and though samples were stored dry to prevent deterioration, the method varied, and soil was stored at 4^{o}C and residue at *ca* 22^{o}C. Therefore, further studies are required to examine the effect of methodology and time for which samples are stored on pathogen survival.

Filtrate from infested soil was subjected to a sieving-column to enable suspensions with known range of particle size to be inoculated onto faba bean plants. The results suggested that

the size of propagules of *C. zonata* that infect faba bean was predominantly 15 to 105 μm. Fractions containing particulates outside this range resulted in significantly fewer lesions on plants. Extracted propagules were not examined further, but the size range does correspond with the description of conidiophores (25-60 μm) and conidia (30-150 μm) of *C. zonata* (Williams, 1987). However, the survival structures of *C. zonata* residing in soil are not known and there is little information on the life cycle of this pathogen, so further research is warranted that could provide valuable information on pathogen survival.

The bioassay utilised in this study may provide a useful method for evaluating the infectivity of infested residue and soil samples as a source of inoculum for CLS on faba bean. However, low disease levels observed in the bioassay tests conducted 12 months after samples were collected, approximately 10 lesions per plant on average in the most diseased treatments, suggests that this technique may be limited as a means to estimate the infectivity of samples containing low or variable levels of CLS inoculum. Therefore, a more sensitive method of quantifying inoculum in infested soil and stubble would be advantageous in further studies to establish the viability of *C. zonata* in infested soil or residue over time.

The studies reported here offer an insight into the biology of *C. zonata* and demonstrate the importance of soil-borne inoculum in development of CLS on faba bean. These preliminary studies showed that the relative contribution of soil-borne and residue-borne inoculum to the primary source of inoculum for CLS development requires further examination, and should include survival time and dissemination of *C. zonata* from these sources. Such research is critical to understand the life cycle of *C. zonata*, particularly to elucidate what propagules of the pathogen reside in soil and lead to primary infection on faba bean. Once primary infection agents are identified the mechanisms of infection can also be studied. The results in this study indicate that the influence of time and cultural practices on survival of CLS inoculum in soil should be scrutinised further to identify management strategies that may reduce the impact of CLS in commercial faba bean crops.

Chapter 5.

Host range, prevalence and management of cercospora leaf spot (*Cercospora zonata*) of faba bean (*Vicia faba*) in southern Australia

Manuscript prepared for submission to *Phytopathology*, except that citations are in the form of author and date, rather than numbers, to be consistent with the format used elsewhere in this thesis.

Host range, prevalence and management of cercospora leaf spot (*Cercospora zonata*) of faba bean (*Vicia faba*) in southern Australia.

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ABSTRACT

Kimber, R. B. E., Paull, J. G., Scott, E. S., and Davidson, J. A. 2011. Host range, prevalence and management of cercospora leaf spot (*Cercospora zonata*) of faba bean (*Vicia faba*) in southern Australia. Phytopathology X:X-X.

The distribution and occurrence, host range and management of *Cercospora zonata*, the causal pathogen of cercospora leaf spot (CLS) of faba bean, in southern Australia were studied. Disease was most severe on faba bean although *C. zonata* did infect and cause lesions on narbon bean, lentil and vetch. *C. zonata* was not pathogenic to other dicotyledonous hosts examined; pea, canola, chickpea, lathyrus or lupin. Host-pathogen

evaluation of other *Cercospora* species which have recently been found in South Australia, *viz*. *C. zebrina* and *Pseudocercosporella capsellae*, is also reported. In a disease survey of 100 commercial faba bean crops in seven districts of southern Australia during 2007, CLS was endemic to all districts but was most severe in the south-east of South Australia. Identified in 87% of crops, disease incidence and severity were highest in fields cropped with faba bean in short rotations (1-4 years) and severity decreased significantly (R^2=0.13, *P*=0.006) as the interval between faba bean crops increased. CLS was also more severe in crops planted adjacent to fields with faba bean residue remaining from the previous year compared to crops that were not adjacent to residues (t=2.57, *P*<0.05). Disease management trials conducted from 2005 to 2007 showed that in favourable conditions, CLS caused severe lesions on foliage and extensive defoliation, and was associated with a 7% reduction in yield. Severe CLS lesions were observed on stems and pods but did not result in stem breakage or obvious effects on seed quality. Applications of carbendazim, tebuconazole, chlorothalonil and triadimefon significantly reduced CLS severity compared with untreated controls. The most cost-effective treatment was one application of carbendazim or tebuconazole to seedlings 5-7 weeks after sowing, prior to disease establishment. This is the first scientific report of the distribution and significance of CLS in faba bean in Australia.

Faba bean (*Vicia faba* L.) has been widely grown in southern Australia since 1980, supported by a breeding program established at the University of Adelaide (South Australia) in 1976 (Knight, 1994). Australia is the world's fifth largest producer (217,000 t) and second largest exporter of faba beans (139,000 t). The majority of production is in the Mediterranean-type environments of southern Australia, predominantly in the states of South Australia (110,000 t) and Victoria (35,000 t) (FAO, 2011; ABARES, 2011).

Cercospora Fresen. is one of the largest and most heterogenous genera of hyphomycetes worldwide and includes over 3,000 species. Allied genera, such as *Cercosporella*, *Cercosporidium*, *Pseudocercospora,* and *Pseudocercosporella,* are often included within a broad assemblage of taxonomic classification referred to as the *Cercospora* complex (Goodwin *et al*., 2001). Cercospora leaf spot (CLS), caused by *Cercospora zonata*, is ubiquitous in many countries where faba bean is cultivated. However, few report this disease as a major concern to faba bean production, and consequently there are very limited critical reviews that address its distribution, significance or the life cycle of *C. zonata* (Woodward 1932; Yu 1947). However, anecdotal evidence suggests that CLS has increased in prevalence and severity in commercial crops in southern Australia since 2004, contrasting with the situation in other countries where faba bean is commonly grown. In addition, in 2004 two other diseases were observed for the first time in South Australia; cercospora blackstem of burr medic (*Medicago polymorpha*), caused by *C. zebrina*, and grey leaf spot of canola (*Brassica napus*) caused by *Pseudocercosporella capsellae* (C Wilmshurst, *personal communication*).

C. zonata is reported to have a relatively limited host range, confined to *Vicia* species such as *V. faba*, *V. narbonensis* (narbon bean) and *V. sativa* (vetch) (Williams, 1987a). Yu (1947) stated that the fungus was not able to infect the following; *Vicia sativa* L., *V. cracca* L., *V. villosa* Roth., *Pisum sativum* L., *Lens esculenta* Moench (*L. culinaris*), *Lathyrus odoratus* L. and *Medicago sativa* L. However, this was contradicted in later studies, where *C.*

zonata was reported to infect *L. odoratus* and *L. culinaris* (Chupp, 1954; Williams, 1987a). Cross-infection sometimes occurs following inoculation with isolates of *Cercospora* spp. from different host genera and species, but isolates are usually most virulent on the host from which they were isolated (Nelson and Campbell, 1990). However, information on severity of disease on alternative hosts is important to establish the role of alternative hosts in the pathogen's life cycle. Vetch and lentil are commonly grown in the cereal belt of southern Australia, as part of regular rotation schedules that also include faba bean. Barbetti (1985) stated that the broad host range of *C. zebrina* was likely to influence the spread of cercospora black stem in pasture legumes in Western Australia. Likewise, Lartley et al. (2005) reported that *C. beticola* isolated from sugar beet fields in the mid-west United States of America also caused leaf spots on safflower crops and recommended that this crop should not be grown in rotation with sugar beet, being an alternative host for the pathogen. Findings such as these can affect management decisions based on rotation with non-hosts as a means of reducing disease carry-over.

C. zonata is presumed to persist in crop residue, either as dormant stromatic mycelium or as clusters of conidiophores (fascicles), remaining on the soil surface, to infect emerging faba bean plants in subsequent seasons (Yu, 1947; Walker, 1952). The current recommendation for Australian farmers is to maintain approximately 4 years between faba beans crops within one field, by which time residues harboring survival structures of other major pathogens of faba bean (*Ascochyta fabae, Botrytis fabae*) have typically decomposed and no longer represent a significant source of inoculum (Wallen and Galway, 1977; Dyke and Prew, 1983). The distribution of CLS in commercial crops in southern Australian has not been established and the persistence of inoculum of *C. zonata* on infested faba bean debris or in soil is not known, so that crop rotations for management of this disease cannot be recommended.

The impact of CLS on faba bean yield is not known. All faba bean cultivars currently adopted by the Australian industry are susceptible to CLS and resistant genotypes have only recently been reported (Kimber & Paull, 2010). Furthermore, there are no reports of fungicide efficacy that can assist the industry in disease management until resistant cultivars become available. Anecdotal information suggests that foliar applications of mancozeb, a fungicide routinely used by Australian growers to control fungal diseases in faba bean, failed to control CLS in recent years. This situation has been further complicated by incorrect diagnosis of disease leading to inappropriate use of fungicides. Recommendations of fungicide applications for disease management require an understanding of efficacy against the target pathogen and the yield penalty in the absence of control. Furthermore, strategies for application of effective and economical control of the disease need to be identified.

The objectives of this study were to (i) assess the specificity of *C. zonata, C. zebrina* and *P. cercosporella* to *V. faba*, *Medicago* spp. and *B. napus*; (ii) elucidate the host range of *C. zonata* on major dicotyledon hosts grown in southern Australian cropping regions; (iii) determine the distribution and significance of CLS in major growing districts of southern Australia; (iv) examine the impact of CLS on yield; and (v) identify effective fungicides and application strategies for control of the disease in faba bean.

MATERIALS AND METHODS

Host specificity of *Cercospora* spp. The pathogencity of *Cercospora zebrina* (isolate 51/04), *C. zonata* (isolate 69/04) and *Pseudocercosporella capsellae* (isolate 29/04) was examined on three hosts; medic (*Medicago* spp.), faba bean (*Vicia faba*) and canola (*Brassica napus*). Isolates were obtained from a working collection at the South Australian Research and Development Institute (SARDI), stored as pure cultures in sterile water at 4^{o}C. The experiment was arranged as a randomised split plot design with four replicates. The main blocks were cultivars: cvs Fiord, Farah and Cairo (faba bean); cvs Santiago (burr clover, *M.*

polymorpha), Sava (snail medic, *M. scutellata*) and Caliph (barrel medic, *M. truncatula*) (medic); and cvs Rivette, 44C11 and Kimberly (canola). Sub-blocks were three fungal species; *P. capsellae, C. zebrina*, and *C. zonata*. Thirty-six trays each containing 12 punnets (550 ml per punnet) were filled with University of California (UC) potting mix (pH 6.3). Twelve trays were sown with each host, and each cultivar randomly assigned to a row of four punnets, with three seeds per punnet sown to a depth of 3 cm then thinned to a single plant per punnet before inoculation. When seedlings were 4 weeks old, four trays of each host were inoculated with 800 ml of suspension of each test isolate, consisting of 10^6 mycelial fragments per ml plus 0.05% surfactant (Tween 20, BDH Laboratory Supplies), applied until run-off. This inoculation method was used as spores of *C. zonata* are not produced in culture (Kimber and Paull, 2010). Treatments within each replicate were separated by acrylic partitions. Immediately after inoculation, plants were placed in a glasshouse fitted with automated ultra-fine misters overhead to produce high humidity conditions and maintained at 18^{o}C (±4^{o}C). Disease was assessed 14 days after inoculation (DAI), as total % disease (%TD), percentage leaf area diseased (%LAD) on mid-canopy leaves, percent defoliation (Defol) and percent stems per plant with lesions (%SL). Results were analysed in Genstat® version 11 using general analysis of variance (ANOVA) and data were transformed (square root) when required to normalize the data. Untransformed data was presented in figures for clarity. Samples of tissue from leaf lesions were taken from each host-pathogen combination, incubated at room temperature in humidity chambers, and resultant conidiophores protruding from lesions were cultured on potato dextrose agar (PDA), to fulfil Koch's postulates for proof of pathogenicity. The identity of the isolates was confirmed using morphological descriptions of pathogenic fungi (Williams, 1987a; Williams, 1987b; Kirk, 2004).

Host range of *C. zonata*. In a preliminary experiment conducted in a controlled environment *C. zonata* did not infect canola (*B. napus*), chickpea (*Cicer arietinum*), lathyrus (*Lathyrus sativus*), pea (*Pisum sativum*), narrow-leaf lupin (*Lupinus angustifolius*) or albus

lupin (*L. albus*). However, symptoms were observed on faba bean (*V. faba*), lentil (*L. culinaris*), vetch (*V. sativa*) and narbon bean (*V. narbonensis*). Therefore, an experiment was conducted in a controlled environment room (CER) to examine the severity of *C. zonata* on these latter four hosts.

The experiment was arranged as a split-plot design with three replicates, whereby host formed main plots and host cultivar the sub-plots, and included two cultivars each of faba bean (cvs Farah and Fiord), lentil (cvs Northfield and Nugget) and vetch (cvs Blanchfleur and Morava), and one cultivar of narbon bean (cv. Tanami). Eighteen trays, each with 12 punnets (550 ml per punnet), were filled with UC potting mix. Three trays were sown for each host species, six punnets for each cultivar. Two seeds of lentil, vetch and narbon bean, or one seed of faba bean were sown in each punnet. For controls, one tray was included in each replicate containing one cv. of each host (cvs Farah, Tanami, Morava and Northfield), each sown in three punnets. Plants were maintained at 15^{o}C with 12 h day-length periods. After 4 weeks, seedlings were inoculated with *C. zonata* using 1500 ml of a mixed-isolate suspension (isolates 37/04, 69/04, 70/04, 71/04, 72/04, 73/04, 74/04 and 77/04) consisting of 10^6 mycelial fragments per ml plus 0.05% surfactant, applied until runoff, whereas control plants were sprayed with RO water plus surfactant. Following inoculation, seedlings were placed in plastic tents in the CER with ultrasonic humidifiers, to maintain leaf wetness and maintained at 15^{o}C. After 10 days, the humidifiers were switched off and tents opened to allow air-flow. Disease was assessed on individual plants 28 DAI by recording; percentage severity, percent LAD of mid-canopy leaves, percent defoliation and the percentage of stems exhibiting lesions, as above. Samples of leaf lesions were taken from plants exhibiting symptoms, incubated in humidity chambers and identified as described above. The results were analysed using ANOVA, performed in Genstat® version 11, and data were transformed (square root) to normalise data when required. Untransformed data are presented in figures for clarity.

Disease Survey 2007. A survey of 100 commercial faba bean crops in southern Australia was conducted between late winter (2 August) and early spring (4 September) of 2007. Seven growing districts within South Australia and Victoria were included (Fig. 1). Each district was defined by different climatic conditions and soil type, and in total they represent *ca* 75% of Australia's faba bean production area. Crops were selected in consultation with local agricultural consultants to represent the broad spectrum of crop management options and rotation history adopted by southern Australian faba bean growers. The following information was recorded for each crop; date, global positioning coordinates, grower's name, field name and crop growth stage. Detailed information was obtained from growers via local consultants for 87 of the 100 crops, including; cultivar, sowing date, year of previous faba bean crop (rotation), proximity to faba bean crop residue remaining from 2006, fungicide application (date, active ingredient and rate) and yield. In each field, 20 plants were collected on a W-shape transect, taking one plant every 10 m (Davidson et al., 2001). Plants were labelled and stored in a cooler box. In the laboratory, incidence (%) and severity (%) of CLS and defoliation (%) were assessed for each plant. Similar data were also recorded for ascochyta leaf blight (*Ascochyta fabae*) and chocolate spot (*Botrytis fabae*), but will not be presented in detail.

Data were analysed using Genstat® version 11 and GraphPad Prism® version 5. The incidence and severity of CLS were regressed against rotation data. Some agronomic information provided by growers for the rotation period was descriptive, therefore conservative values were assigned for these data, *viz*.; 'never' = 20 years, 'over 10 years' = 12, '5-10 years' = 7. The association between disease severity and growing region, rotation and proximity to faba bean residues was investigated using chi-squared (χ^2) and two-tailed t-test (t) analyses. Significant differences were separated at $P<0.05$.

Fungicide treatments for CLS. The effect of foliar fungicide applications on CLS was evaluated in field trials conducted in 2005, 2006 and 2007 at Charlick Research Station near

Strathalbyn, South Australia. Each trial was designed as a completely randomised block with four replicates. Plots were 6 x 1.6 m and sown with six rows at 25 cm spacing. There were two untreated control plots per replicate, and an untreated buffer plot between adjacent treatment plots. Cultivar Farah, which is susceptible to CLS (Kimber and Paull, 2010) but resistant to ascochyta leaf blight, was sown at planting density 25 plants/m^2. Natural infection was encouraged by locating trials in fields with a recent history of faba bean (3-4 years since last crop). Foliar fungicides were applied using an electric hand-held sprayer (custom-built, SARDI Crop Evaluation) at 2 bar pressure and fitted with a 1.7-m boom with four Hardi® flat-fan nozzles operated approximately 0.4 m above the crop canopy. In 2005 and 2006, flat fan No.10 nozzles (size 4110-10 fan jet or ISO F-01-110) were used to deliver an application rate of 200 liter/ha. In 2007, low drift nozzles were used (flat fan No.12, LD-110 015-Green), to optimise fungicide coverage, at an application rate of 247 liter/ha. Table 1 provides descriptions of treatment rates and timing in each trial. Weather data were recorded on site each year using an Automated Weather Station (Measurement Engineering Australia), *viz*.; rainfall, temperature (^oC), relative humidity (%), leaf wetness (%), and soil temperature (^oC) at daily and 15 min intervals.

The 2005 trial was designed to evaluate the efficacy of seven foliar fungicides (Table 1; treatments T1-T7) registered for use in pulse crops at label recommendations (Hawthorne et al., 2005). The trial was sown on 29 June and treatments commenced 7 weeks after sowing (WAS) on 17 August, with subsequent applications every 3 weeks (at 10, 13 and 16 WAS) on 8 and 27 September and 20 October, respectively. On 9 November (19 WAS), a mixture of procymidone (Sumisclex® at 500 ml/ha) and chlorothalonil (Bravo720® at 2.0 liter/ha) was applied once to all plots to prevent the development of chocolate spot (*Botrytis fabae*). Symptoms of CLS were recorded at 12 WAS (20 September) and 18 WAS (4 November) as; incidence per plot (%), disease severity per plot (%), leaf area diseased on 10 randomly sampled leaf-pairs (%LAD) in the lower canopy and the incidence of defoliation (%) per plot.

The incidence and severity (%) of CLS lesions on pods was assessed 21 WAS (21 November). Trial plots were harvested on 10 December and yield data per plot recorded as t/ha. Sub-samples of 100 seeds from each plot were weighed and assessed for discolouration or staining.

The 2006 trial was established to evaluate the efficacy of eight foliar fungicides and of a single application of carbendazim at 7 or 10 WAS (Table 1; treatments T1-T5, T8-T10 and S1-S2). The fungicides were chosen for their broad spectrum activity and low cost of treatment. The trial was sown on 11 June and treatments commenced 7 WAS (4 August), with subsequent applications (if scheduled) at 10, 13 and 16 WAS on 25 August, 15 September and 6 October, respectively. Treatment T8 (Captan) commenced at 10 WAS due to delayed availability of product. Due to severe drought, the trial was assessed for CLS only once at 14 WAS (21 September), during flowering and following two fungicide applications. Disease was recorded as %CLS severity (incorporating defoliation) per treatment plot. Trial plots were harvested on 28 November and yield data recorded as t/ha for each plot.

The 2007 trial was established to evaluate four foliar fungicides using seven application strategies (Table 1; treatments T1-T3, S1 and S3-S6), based on results from field trials conducted in 2005 and 2006. The trial was designed to compare (i) different fungicides and rates using a single application at 7 WAS (30 July); (ii) the application of a fungicide 7 WAS and 5 WAS (13 July); and (iii) a single application of fungicide and two applications made at 7 and 10 WAS (18 Aug), or four applications made at 7, 10, 13 WAS (7 September) and 16 WAS (4 October). Disease severity (%) per plot was assessed only once, due to drought conditions, at 13 WAS (7 September) during flowering following two fungicide applications. Defoliation (%) was recorded at 18 WAS (19 October).

Analysis of results. Disease and yield data were analysed using a generalised analysis of variance (ANOVA), performed in Statistix® for Windows version 8.0. Treatments were pooled into low (<25%) and high (>50%) disease categories, separated by significance

(P<0.05), using assessments taken at 18 WAS, and ANOVA was performed on the yield data of pooled treatments.

RESULTS

Host specificity of *Cercospora* spp. Inoculation of faba bean, medic and canola cultivars with *C. zonata*, *C. zebrina,* and *P. capsellae*, respectively, resulted in lesions 14 days after inoculation. Emergence of seedlings of canola cv. Kimberly was poor so the data were omitted from the analysis.

C. zonata caused severe disease on two of the three cultivars of faba bean and slight disease on barrel medic (*M. truncatula*, cv. Caliph) (Fig. 2). Severe disease, in terms of leaf lesions, defoliation and stem lesions (means >70% TD, 30-60% Defol and 16% SL), was observed on cvs Farah and Fiord, and moderate disease with few stem lesions (means = 27% TD, 22% Defol and 1% SL) on cv. Cairo, where individual plants exhibited resistant or susceptible responses. Conidia collected from incubated symptomatic leaf tissue confirmed the identity of the pathogen as *C. zonata,* satisfying Koch's postulates (Table 2). Slight disease (P<0.05; means = 3% TD, 2% LAD and 5% Defol) was evident on medic cv. Caliph. No sporulation was observed on leaf lesions of cv. Caliph and attempts to isolate fungi failed to confirm a casual pathogen (Table 2).

C. zebrina caused varied disease, ranging from severe to slight, on the three cvs of medic, and slight disease on faba bean (Fig. 2). Severe disease (means = 82% TD, 47% Defol and 76% SL) was observed on snail medic cv. Sava, moderate disease (means = 27% TD, 15% Defol and 7% SL) on barrel medic cv. Caliph, and slight disease (means = 9% TD, 9% Defol and 5% SL) on burr clover cv. Santiago. Conidia collected from incubated leaf lesions confirmed the identity of the pathogen as *C. zebrina* (Table 2). Minor specking on leaves and stems (P<0.05; means = 5% TD and 1% SL) was observed on the faba bean cvs, expressed as

restricted lesions (< 2mm). No sporulation was observed on incubated leaf lesions and attempts to isolate fungi failed to confirm a casual pathogen (Table 2).

P. capsellae caused slight disease on canola and two of the three cultivars of medic (Fig. 2). Leaf lesions and defoliation were observed on canola cvs 44C11 and Rivette (means = 14% TD, 10% Defol) and medic cvs Caliph and Sava ($P<0.05$; means = 6% TD, 9% Defol). Conidia collected from incubated leaf lesions confirmed the identity of the pathogen as *P. capsellae* (Table 2).

Host range of *C. zonata.* Inoculation with *C. zonata* caused symptoms on faba bean, narbon bean, lentil and vetch. Control plants did not exhibit symptoms of disease. At 28 DAI mean CLS and defoliation on faba bean plants was 55% and 30%, respectively, which was significantly more severe ($P<0.05$) than disease on the other hosts (Fig. 3). Disease severity on lentil, vetch and narbon bean varied amongst host species and genotypes, ranging from 5 to 23% CLS and 1 to 10% defoliation, however these variations were not significantly different ($P>0.05$) from one another. Stem lesions, where approximately 10% of stems were affected, were observed only on genotypes of faba bean (data not shown).

The pathogenicity of *C. zonata* to faba bean, narbon bean, lentil and vetch was confirmed. *C. zonata* sporulated most prolifically on incubated leaf lesions on faba bean. Leaf lesions exceeding 10 mm in diameter exhibited fasciculate bundles of conidia protruding from about 30% of their surface. Leaf lesions on narbon bean, lentil and vetch were smaller than 10 mm in diameter and only 10% of the surface of lesions on narbon bean leaves and 5% of that on lentil and vetch leaves bore conidia. Conidia were 30-150 μm long and 3.5-5.5 μm wide, consistent with morphological descriptions of *C. zonata* (Williams, 1987a), and growth of mycelium in culture from conidia transferred from lesions on each host was consistent with growth characteristics of *C. zonata.*

Disease survey 2007. Cercospora leaf spot was present in 87 of the 100 commercial crops inspected in 2007 (Table 3) and was present in all seven districts. By comparison,

substantially fewer crops were affected by ascochyta leaf spot (48) or chocolate spot (6). Of the 87 crops for which agronomic information was available, Fiesta was the most widely adopted cultivar (51), followed by cvs Farah (19), Nura (8), Aquadulce (6) and Fiord (3). In these crops, 67 were sown in fields with a history of faba beans (≤12 years) and 20 were sown in fields that had never been used to grow faba bean. The most common interval between faba bean crops adopted by growers was 3-4 years, occurring for 21 crops. Crop yield ranged from 0.08 to 6.1 t/ha across all districts although the data set is incomplete because not all growers recorded yield accurately. The largest district mean yield (3.5 t/ha) occurred in the high-rainfall districts of the lower south-east of South Australia. Yield was not correlated with CLS severity, crop rotation or sowing date and variability in the data as drought conditions confounded interpretation of the data.

Differences in the incidence and severity of CLS were observed in the seven districts surveyed (Fig. 4). Significantly greater disease incidence (t=2.6. df=23, $P<0.05$) and severity (t=3.54, df=20, $P<0.05$) was observed in the two south-east districts (incidence = 76.2 ± 9.1, severity = 39.5% ± 5.8, N=17) than in the five other growing districts (incidence = 50.1% ± 4.3, severity = 17.6% ± 2.0, N=83) of South Australia and the Victorian Wimmera. This higher incidence and severity of CLS in the SE districts was not attributed to closer faba bean rotations adopted within these districts since similar frequencies of high risk rotations (1-4 year rotation) were observed in all seven districts (χ^2=17.7, df=12; P=0.13) (Fig. 5). Additionally, the greatest frequency of crops sown in high risk fields was observed in the Mid-North of South Australia where CLS was least severe. However, few crops were within the low risk category (>9 year rotation) in the south-east districts.

The history of faba bean plantings had a significant effect on the incidence and severity of CLS for all surveyed crops (Fig. 6). The incidence of disease observed in surveyed crops significantly decreased (R^2=0.12, $P<0.01$) with longer interval between faba bean crops. This relationship also applied to disease severity (R^2=0.13, $P<0.05$), where a significant (t=3.87

df=22, $P<0.001$) increase in CLS severity (38.5% LAD ± 5.6, N=19) was observed in crops planted in 1-4 year rotation fields compared with CLS severity (15.5% LAD ± 2.0, N=68) observed in crops planted in fields with a rotation of more than 5 years. Furthermore, the mean disease severity was significantly (t=2.57, df=85; $P<0.05$) greater in crops (28.3% ± 4.0, N=29) grown adjacent to faba bean residues remaining from the previous season (2006) than in crops (16.6% ± 2.5, N=58) not adjacent to these residues (Fig. 7).

Fungicide treatments for CLS. In the 2005 field trial, CLS lesions appeared on seedlings 7 WAS, when plants were at the 4 to 6 leaf node stage and were visible when foliar fungicides were first applied. Lesions first appeared on the lowest leaves of seedlings distributed evenly throughout the trial site, and developed to the point where plants exhibited severe lesions on leaves, stems and pods to 66% severity in untreated plots at 21 WAS. At 12 and 18 WAS there were significant differences ($P<0.05$) among treatments in terms of fungicide efficacy against CLS (Fig. 8). Plots that received four applications of carbendazim, commencing 7 WAS and repeated every 3 weeks, showed the least disease (average CLS 8% per plant). Disease severity remained low in plots treated similarly with chlorothalonil and tebuconazole (20 and 22%, respectively) and was significantly ($P<0.05$) less than in other treatments. Defoliation was also significantly ($P<0.05$) lower in plots treated with carbendazim (8%), chlorothalonil (11%) and tebuconzole (14%) than untreated plots (37%). Disease on plants treated with cuprous hydroxide, mancozeb, procymidone and copper oxychloride exhibited similar severity to the untreated controls, with 53-66% CLS and 22-37% defoliation. At 21 WAS, significant differences ($P<0.05$) were also identified in the incidence of CLS on pods. Plots treated with carbendazim showed fewest pod lesions per plot (5%$P<0.05$). Pod infection was also significantly ($P<0.05$) reduced in plots treated with chlorothalonil and tebuconazole (68 and 77%, respectively), compared with plots treated with cuprous hydroxide, mancozeb, procymidone and copper oxychloride (92-95%), all of which exhibited incidence of pod lesions similar to untreated plots (99%).

Cercospora leaf spot had no effect (P>0.05) on 100-seed weight or seed quality. Negligible blemishes were visible on seed and no differences in staining or discolouration were evident among treatments. Yield ranged from 3.5 to 4.2 (± 0.2) t/ha across treatments. Yield data for treatments that resulted in high and low CLS severity at 18 WAS were pooled separately (Fig. 8), and analysed using ANOVA with unbalanced randomised complete block design. Yield was reduced (P<0.05) by 7% in treatments exhibiting high CLS (3.68 ± 0.07 t/ha) compared with treatments with low CLS (3.95 ± 0.10 t/ha).

In the 2006 field trial, CLS lesions appeared on faba bean seedlings 7 WAS and developed to 52% severity in untreated plots by 14 WAS. Severe drought conditions after this period suppressed plant growth and interfered with disease spread. Disease assessment at 14 WAS, a week after the third application of repeated fungicide treatments (applied 7 WAS and repeated every 3 weeks), showed significant (P<0.05) treatment effects (Fig. 9). Regular applications of carbendazim, chlorothalonil, tebuconazole and triadimefon reduced (P<0.05) CLS severity (≤26% CLS) compared with untreated controls; tebuconazole resulted in the least disease (5% CLS). Disease severity on plants treated only once with carbendazim, applied at 7 or 10 WAS, was 13 and 29%, respectively, similar to repeated applications and significantly (P<0.05) less than untreated controls. Plants treated with mancozeb, metiram, procymidone and captan showed no significant (P>0.05) reduction in CLS severity compared with untreated plots. Further disease assessments were not possible, due to severe drought, and yield was severely depressed (mean yield 0.37 t/ha), thus yield data were not amenable to statistical analysis.

In the 2007 field trial, CLS lesions first appeared at 7 WAS on the lowest leaves of seedlings and increased to 49% CLS and 30% defoliation in untreated plots by 14 WAS. Drought, though not as severe as 2006, again suppressed plant growth and disease spread. At 13 WAS disease assessments showed a significant (P<0.05) treatment effect (Fig. 10). All foliar fungicide treatments significantly reduced disease (<30%) compared with untreated

controls (48%). CLS severity was comparable among all treatments using chlorothalonil, carbendazim or tebuconazole (<13%), whether applied once or twice prior to assessment. There was no significant difference between recommended (145 ml/ha) and high (290 ml/ha) application rates of tebuconazole applied at 7 WAS. Disease on plants treated once with triadimefon at 7 WAS, at recommended (500 ml/ha) or high (1000 ml/ha) application rates, was more severe (29% CLS) than that for other fungicide treatments (<14% CLS), including two applications of triadimefon at recommended rate (500 ml/ha) 7 and 10 WAS (14% CLS). At 18 WAS, spread of CLS had slowed and defoliation was the main symptom in the trials, exacerbated by water stress. Plants subjected to all treatments using triadimefon, or a single application of tebuconazole at 145 ml/ha 7 WAS, exhibited moderate defoliation (26-33%) and were not significantly different from untreated controls (30%). However, single applications of carbendazim (5 or 7 WAS) or tebuconazole at 290 ml/ha (7 WAS), and four applications of carbendazim, tebuconazole or chlorothalonil showed significantly (P<0.05) less defoliation (8-20%) than the untreated controls.

The grand mean yield of the 2007 trial was 1.5 t/ha. While there was no significant effect of CLS on yield, since drought compromised data for analyses, there was a trend whereby faba bean yield was reduced by 3% when CLS was severe, i.e. the mean yield of treatments exhibiting high CLS levels (≥20% defoliation) was 1.58 t/ha (± 0.06) compared with 1.63 t/ha (± 0.06) for treatments with little CLS.

DISCUSSION

C. zonata, C. zebrina and *P. capsellae* were pathogenic to the host from which they were isolated; *V. faba*, *Medicago* spp. and *B. napus,* respectively, as previously reported (Yu, 1947; Barbetti, 1985; Inman et al., 1997). Furthermore, minor disease was caused on medic by *P. capsellae* and on faba bean by *C. zebrina* in controlled environment experiments. An absence of sporulation by *C. zebrina* on lesions on faba bean leaves prevented isolation in our

experiment but the pathogenicity of *Cercospora* spp., isolated from clover, to several legume genera including *Vicia* spp. has been previously reported, though species were not named (Berger and Hanson, 1962). To our knowledge, this is the first report of pathogenicity to medic of *P. capsellae*, a fungus known to cause white leaf spot disease on many brassica crops and several cruciferous weeds (Petrie and Vanterpool, 1978; Eshraghi et al., 2007). However, further research is required to confirm if this fungus will infect *Medicago* spp. in the field.

C. zonata caused slight disease on barrel medic, narbon bean, lentil and vetch in controlled environment experiments. Pathogenicity to each host, except medic, was confirmed by culturing the fungus from tissue excised from sporulating leaf lesions. *C. zonata* did not infect pea, canola, chickpea, narrow-leaf lupin, albus lupin or lathyrus in preliminary experiments, in contrast to earlier reports that *C. zonata* was pathogenic to lathyrus but not to lentil and vetch (Yu, 1947; Chupp, 1954; Williams, 1987a). The severity of disease and fecundity of *C. zonata* on alternative hosts in this study was significantly less than that observed on its primary host, faba bean, which supports results from cross-infection studies for other cercospora diseases (Nelson and Campbell, 1990). Though reduced severity of CLS was observed in faba bean cv. Cairo, compared with cvs Farah and Fiord, this can be attributed to heterogeneity in reaction to the disease in this genotype (Kimber and Paull, 2010). Furthermore, variation in aggressiveness was also observed on vetch and lentil cultivars, suggesting genetic control of resistance may also exists in these hosts. This may explain the conflicting reports that lentil and vetch are hosts of *C. zonata*, as results could be affected by selection of host genotype.

The importance and ecological role of alternative hosts for *C. zonata* has not been examined critically. It is unclear whether the existence of alternative hosts of *C. zonata* in an environment where those hosts are in close proximity offers an evolutionary advantage or whether crossing the species barrier itself may lead to further specialisation (Woolhouse et al.,

2001). The ability of *C. zonata* to colonize vetch and lentil may influence pathogen survival, fitness or aggressiveness in passage through these alternative hosts. This effect has been demonstrated in studies of population dynamics of other host-pathogen systems (Regoes et al., 2000; Zhan et al., 2002; Vizoso and Ebert, 2005). However, the biological significance of the ability of a *Cercospora* species to infect different hosts via artificial inoculation should not be overstated, particularly if the pathogen has yet to be isolated from that host in the field (Groenewald et al., 2006). This requirement should be considered in future studies of *C. zonata,* including pathogenicity studies on other species of *Vicia* that occur as weeds in southern Australia, and may offer insight into the recent increase in prevalence of CLS in southern Australia.

CLS was endemic in the major faba bean growing districts of southern Australia and was the most prevalent disease observed in the commercial crops surveyed in 2007. This finding supports recent observations that CLS has increased in this region since 2004 and is the first report in the world of severe and widespread epidemics of CLS in commercial crops of faba bean. Climate data for most growing regions in South Australia and Victoria during August and October of 2007, when the survey was conducted, showed below average rainfall compared to the long-term average data. However, the 2007 season was consistent with a trend that was applicable for climate data recorded between 2002 and 2009, during which most faba bean production regions experienced below average rainfall and above average temperatures during the growing season compared to the long term average (BOM, 2010). Therefore, further research on the effects of temperature and wetness periods on CLS are warranted.

Crop rotation was shown to be an important factor in the development of a CLS epidemic. The disease was more frequent and severe on plants grown in fields with short rotations of faba bean (1-4 years), and severity decreased significantly with extended intervals between faba bean crops. This suggests a link to localised soil-borne inoculum, promoted by

short rotation periods, which has also been reported for other cercospora diseases (McKay and Pool, 1918; Ward et al., 1999; Vereijssen et al, 2006). In addition, CLS was more severe in crops planted adjacent to fields containing faba bean residue remaining from the previous season, a potential source of CLS inoculum, than in crops that were not adjacent to these residues. This indicates that inoculum may also be disseminated some distance by wind, either as conidia, or as dry-blown particles of infested residue or soil-borne inoculum, as reported for other Cercospora diseases (Carlson, 1967; de Nazareno et al., 1993; Ward et al., 1999). This was also demonstrated by the presence of disease in crops with no history of faba bean or residue in adjacent fields, thus presumed to have originated from infested particles (soil/residue) dispersed by wind from distant fields, a phenomenon reported for CLS of sugar beet (Vereijssen et al, 2006).

CLS severity varied among the growing districts included in this study. Disease was most severe in the south-east districts of South Australia. This variation could not be attributed to an increased frequency of close rotations in that region, but may be affected by the low frequency of crops with long rotations, causing a skewed population distribution. In addition, the south-east region is a long-established production region for faba and broad bean (*V. faba* var. *major*) in Australia and the region in which CLS has been a long-term problem (Wayne Hawthorne, *personal communication*). This suggests that build-up of inoculum in soils of this region is greater than that in other Australian growing regions in which *C. zonata* has become established more recently. As a consequence, if intensive cropping of faba bean continues in these regions, severity of CLS may increase similar to those levels identified in the south-east regions. This effect has been reported in sugar beet production areas of the Netherlands affected by *C. beticola* (Vereijssen et al, 2006). Further research to examine the distribution of *C. zonata* inoculum in soil that includes sampling of fields subjected to different rotation practices, growing regions and residue management of faba bean in southern

Australia is recommended to further our understanding of this disease and the factors that influence build-up of inoculum.

This study is the first to report yield loss in faba bean associated with CLS. In 2005, when long-term average rainfall patterns were experienced, extensive lesions on foliage and defoliation of the lower canopy was associated with a 7% decrease in yield. This effect was evident in a second field trial, in that faba bean yield was reduced by 3% when CLS was severe, but yield was compromised by severe drought. In disease-conducive conditions, CLS caused extensive lesions on stems, but did not girdle or break stems, nor did pod lesions adversely affect seed quality, such as seed size or color of the seed coat. In contrast infection of stems, pods and seed by *Ascochyta fabae* and *Botrytis fabae* contributes to yield loss (Gaunt, 1983). Yield loss in faba bean due to CLS is likely to reflect loss of photosynthetic capacity caused by lesion expansion and defoliation during critical periods of plant growth. This association has been reported for yield loss caused by other *Cercospora* pathogens affecting various hosts (Muro et al., 2001; Proulx and Naeve, 2009). Disease management practices that minimise the loss of photosynthetic leaf area in the early stages of crop development are likely to also minimise yield loss (Karamonos and Gimenez, 1991).

This research has demonstrated that fungicide applications can be used effectively to manage CLS in faba bean. Compared with the untreated control, treatment with carbendazim, tebuconazole, chlorothalonil or triadimefon significantly reduced the severity of CLS when applied at label rates two to four times at intervals of 3 weeks commencing 7 WAS. These fungicides suppressed CLS development and reduced defoliation and, with the exception of triadimefon, have been reported previously to control cercospora diseases of other plants (Wegulo et al., 1998; Galloway, 2008; Culbreath et al., 2009). Furthermore, one application of carbendazim or tebuconazole at the seedling stage, 5 to 7 WAS, resulted in good control, similar to repeated applications of effective fungicides. In consideration of the 3-7% yield loss where disease was not controlled, this strategy represents a cost effective treatment for

control of CLS in faba bean. Our study also showed that mancozeb, a broad spectrum fungicide which is frequently used in faba bean crops to control other foliar pathogens, failed to control CLS in two field trials and confirms anecdotal observations in the field during previous seasons. This contrasts with fungicide efficacy studies on cercospora diseases in other hosts, where mancozeb reduced disease severity (Khan and Smith, 2005; Munkvold et al., 2001; Wegulo et al. 1998), and will offer Australian growers greater confidence in their selection of fungicides to control CLS in faba bean. This study focused on products that are currently registered for use on pulse crops in Australia. Evaluating the efficacy other active ingredients should be considered in future studies. Additionally, triadimefon was found here to be less effective when applied as a single strategic application at recommended or double the label rates and single strategic applications of chlorothalonil were not examined. Strategic applications of these fungicides should be examined to broaden the options for growers to control CLS in faba bean.

This study provides the first report of the distribution and significance of CLS in faba bean in Australia. The increase in prevalence of CLS in recent years may be linked to the accumulation of inoculum within fields in which faba bean has been routinely included, a crop rotation which has became more common in south-eastern Australian over the last three decades. Increased adoption of minimum tillage, which has been associated with an increase in other cercospora diseases that persist on infested residue retained on the soil surface for extended periods, may also be a factor (Ringer and Grybauskas, 1995; Khan et al., 2008). In such situations, yield loss could be minimised by early strategic applications of effective fungicides prior to, or at the initial stages of disease development until cultivars with resistance to CLS become available to growers.

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Table 1: Foliar fungicide treatments applied to faba bean cv. Farah in field trials at Charlick Research Station, South Australia, conducted in 2005, 2006 and 2007 to evaluate efficacy against cercospora leaf spot. Treatments were applied to field plots at the intervals specified.

Treat	Fungicide (a.i.)	Product and application rate	Applied	Repeat application	Year(s) evaluated
T1	Carbendazim	Bavistin® 500 g a.i./liter at 500 ml/ha	7 WAS	Every 3 weeks	2005, 2006, 2007
T2	Tebuconazole	Folicur® 430 g a.i./liter at 145 ml/ha	7 WAS	Every 3 weeks	2005, 2006,2007
T3	Chlorothalonil	Bravo® 720 g a.i./liter at 2.0 liters/ha	7 WAS	Every 3 weeks	2005, 2006,2007
T4	Mancozeb	Dithane® DF 750 g a.i./kg at 2.2 kg/ha	7 WAS	Every 3 weeks	2005, 2006
T5	Procymidone	Sumisclex® 500 g a.i/liter at 500 ml/ha	7 WAS	Every 3 weeks	2005, 2006
T6	Copper oxychloride	County® 500 g a.i./kg at 2.5 kg/ha	7 WAS	Every 3 weeks	2005
T7	Cuprous hydroxide	Kocide BlueXtra® 360 g a.i./liter at 1.65 kg/ha	7 WAS	Every 3 weeks	2005
T8	Captan	Captan® 800 g a.i./kg at 2 kg/ha	10 WAS	Every 3 weeks	2006
T9	Metiram	Polyram® DF 700 g a.i./liter at 2 kg/ha	7 WAS	Every 3 weeks	2006
T10	Triadimefon	Turret® 125 g a.i./liter at 500 ml/ha	7 WAS	Every 3 weeks	2006
S1	Carbendazim	Bavistin® 500 g a.i./liter at 500 ml/ha	7 WAS	-	2006, 2007
S2	Carbendazim	Bavistin® 500 g a.i./liter at 500 ml/ha	10 WAS	-	2006
S3	Carbendazim	Bavistin® 500 g a.i./liter at 500 ml/ha	5 WAS	-	2007
S4	Tebuconazole	Folicur® 430 g a.i./liter at 145 ml/ha	7 WAS	-	2007
S4 high	Tebuconazole	Folicur® 430 g a.i./liter at 290 ml/ha	7 WAS	-	2007
S5	Triadimefon	Turret® 125 g a.i./liter at 500 ml/ha	7 WAS	-	2007
S5 high	Triadimefon	Turret® 125 g a.i./liter at 1000 ml/ha	7 WAS	-	2007
S6	Triadimefon	Turret® 125 g ai.i/liter at 500 ml/ha	7 WAS	10 WAS	2007
Control	No fungicide	-	-	-	2005, 2006, 2007

Treat = treatment code, WAS = weeks after sowing, a.i. = active ingredient

Table 2: Disease symptoms on faba bean (*Vicia faba*), medic (*Medicago* spp.) and canola (*Brassica napus*) following inoculation with *Cercospora zonata, C. zebrina* or *Pseudocercosporella capsellae*. Inoculated plants were maintained in constant high humidity for 14 days and confirmation of pathogenicity is shown by positive (+) or negative (-) results of isolations in culture from diseased plant samples.

Pathogen	Host	Symptoms on foliage	Isolation
C. zonata	*Vicia faba*	Dark brown necrotic lesions, severe coalescence and intense sporulation	+
	Medicago	Infection evident, but lesions restricted and without sporulation	-
	Brassica napus	No symptoms	na
C. zebrina	*Vicia faba*	Infection evident but lesions restricted (<2 mm diam.) and without sporulation	-
	Medicago	Pale brown lesions (≤25mm diam.) with prolific sporulation and stem lesions	+
	Brassica napus	No symptoms	na
P. capsellae	*Vicia faba*	No symptoms	na
	Medicago	Pale brown lesions, some coalescence and tissue collapse	+
	Brassica napus	Pale grey necrotic lesions, small (≤10mm diam.)	+

na = not applicable

Table 3: The number of crops with foliar fungal diseases; cercospora leaf spot (*Cercospora zonata*), ascochyta blight (*Ascochyta fabae*) and chocolate spot (*Botrytis fabae*), identified in 100 commercial faba bean crops surveyed in seven districts within South Australia (SA) and Victoria, Australia in 2007.

Growing District	**Crops surveyed**	**Cercospora leaf spot**	**Ascochyta blight**	**Chocolate spot**
Eyre Peninsula - SA	7	5	1	1
Yorke Peninsula - SA	15	12	0	0
North - SA	12	11	8	0
Mid North - SA	29	24	29	5
Upper South-East - SA	6	5	1	0
Lower South-East - SA	11	11	0	0
Wimmera - Victoria	20	19	9	0
Total number of crops	100	87	48	6

Figure Legends

Figure 1. Location of 100 commercial faba bean crops within the main growing districts of southern Australia which were included in a foliar disease survey conducted during winter and early spring of 2007.

Figure 2. Disease severity (untransformed data) on three cultivars each of faba bean (*Vicia faba*) and medic (*Medicago* spp.), and two cultivars of canola (*Brassica napus*) when inoculated with a mycelial suspension (*ca* 10^5 fragments/ml) of *Cercospora zonata* (isolate 69/04), *C. zebrina* (isolate 51/04) or *Pseudocercosporella capsellae* (isolate 29/04). Inoculated plants were maintained in constant high humidity and assessed 14 days later using percentage scale for Total Disease (TD), Defoliation (Defol), Leaf Area Diseased (LAD) and Stem Lesions (SL). Data were transformed (square root) for general ANOVA: $LSD_{0.05}$ (isolate*cultivar) for TD = 1.16, Defol =1.28, LAD = 1.12 and SL = 1.11.

Figure 3. Cercospora leaf spot (CLS) and defoliation severity on cvs Farah and Fiord of faba bean (*V. faba*), cvs Blanchfleur and Morava of vetch (*Vicia sativa*), cvs Nugget and Northfield of lentil (*Lens culinaris*) and cv. Tanami of narbon (*V. narbonesis*) 28 days after inoculation with a mixed-isolate suspension (10^5 mycelial fragments/ml) of *Cercospora zonata*. $LSD_{0.05}$ CLS = 17.15 and Defoliation = 10.57. Bars indicate standard error.

Figure 4. Mean cercospora leaf spot (CLS) severity observed in plants (20) sampled from 100 commercial faba bean crops surveyed for foliar diseases in 2007 across seven major growing districts of southern Australia: South Australian districts, EP = Eyre Peninsula, YP = Yorke Peninsula, Nth = North, SE = South East; and the Victorian Wimmera. Box plots represent 50% of data, separated by the median, and whiskers extend to the lower and upper 25% of data.

Figure 5. Frequency of commercial faba bean crops affected by cercospora leaf spot, based on incidence data, across seven major growing districts of south-eastern Australia in 2007; data from 75 crops, where the rotation history of those fields was known, were grouped according to number of years since faba bean were last grown in each field surveyed. EP = Eyre Peninsula, YP = Yorke Peninsula, Nth = North, SE = South East.

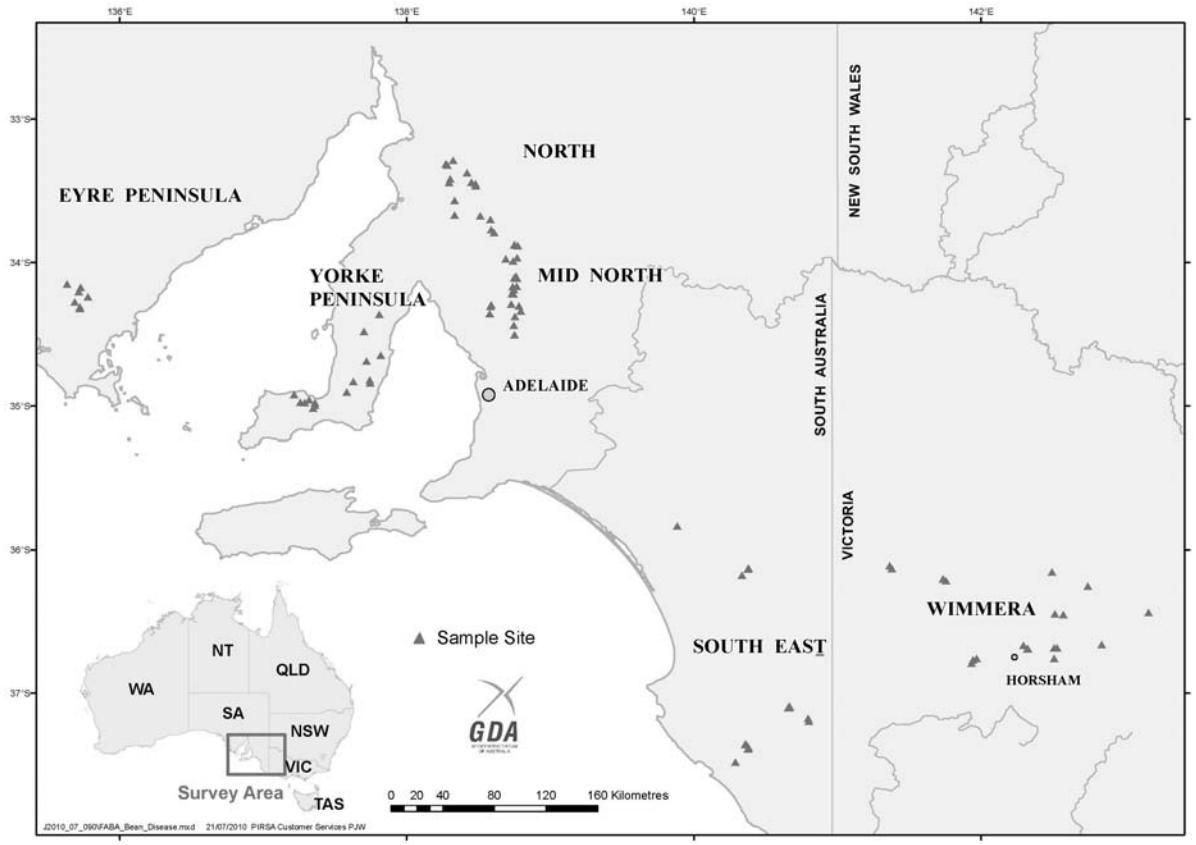
Figure 6. The effect of faba bean rotation on cercospora leaf spot (CLS) severity in 100 commercial crops surveyed across seven major growing districts of southern Australia in 2007. Mean CLS of 20 plants sampled from each field. High CLS risk = 1-4 year rotation of faba bean, lower CLS risk = ≥ 5 years between faba bean crops in the surveyed field. Crops that were reported to be in close proximity to faba bean residues remaining from 2006 are illustrated; ■ = surveyed crop adjacent to faba bean residues, □ = surveyed crop not adjacent to residues.

Figure 7. The effect of proximity to previous faba bean residue (2006 season) on mean cercospora leaf spot (CLS) severity observed on plants (20) sampled from 87 commercial crops included in a foliar disease survey in 2007. Box plots represent 50% of data, separated by the median, and whiskers extend to the lower and upper 25% of data.

Figure 8. Effect of seven foliar fungicide treatments on cercospora leaf spot (CLS) severity assessed 18 weeks after sowing (WAS) in a field trial conducted in 2005 at the Charlick Research Station, South Australia. Treatments were applied four times beginning 7 WAS and repeated every 3 weeks (see Table 1). $LSD_{0.05}$ (CLS severity) = 9.0, $LSD_{0.05}$ (Defoliation) = 10.5. Bars indicate standard error. Broken line boxes indicate statistical (P<0.05) separation of treatments into low and high disease categories to determine the effect of CLS on faba bean yield.

Figure 9. Effect of eight foliar fungicides on cercospora leaf spot severity assessed 14 weeks after sowing (WAS) in a field trial conducted in 2006 at the Charlick Research Station, South Australia. Fungicides were applied three times beginning 7 WAS and repeated every 3 weeks, and strategic treatments of carbendazim were applied once at 7 or 10 WAS (see Table 1). $LSD_{0.05}$ (CLS severity) = 18.8. Bars indicate standard error.

Figure 10. Effect of four foliar fungicides on cercospora leaf spot severity 13 weeks after sowing (WAS) in a field trial conducted in 2007 at the Charlick Research Station, South Australia. Treatments were applied twice at 7 and 10 weeks after sowing (WAS) or once at 5, 7 or 10 WAS (indicated) at recommended or high rates (see Table 1). $LSD_{0.05}$ (CLS severity) = 7.99. Bars indicate standard error.



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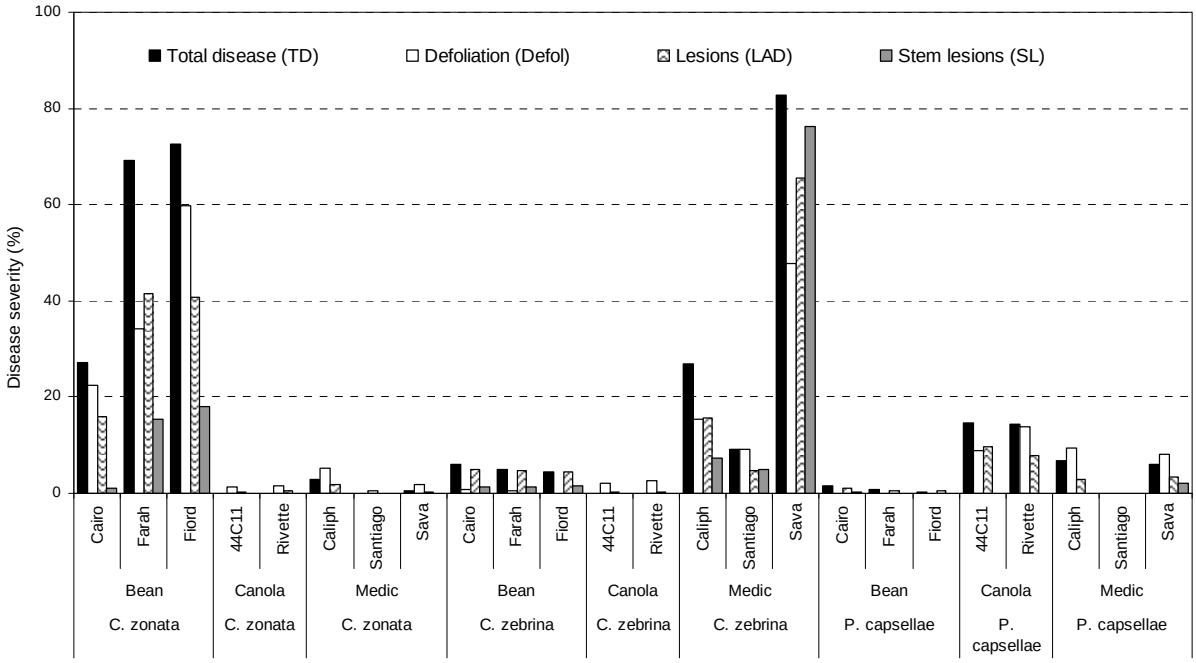

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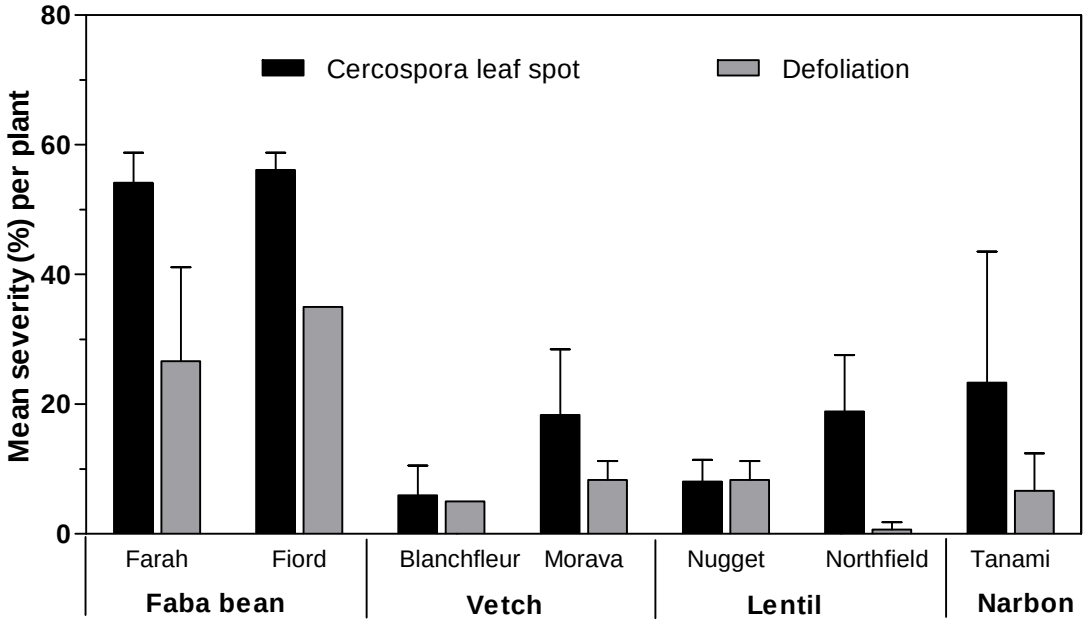

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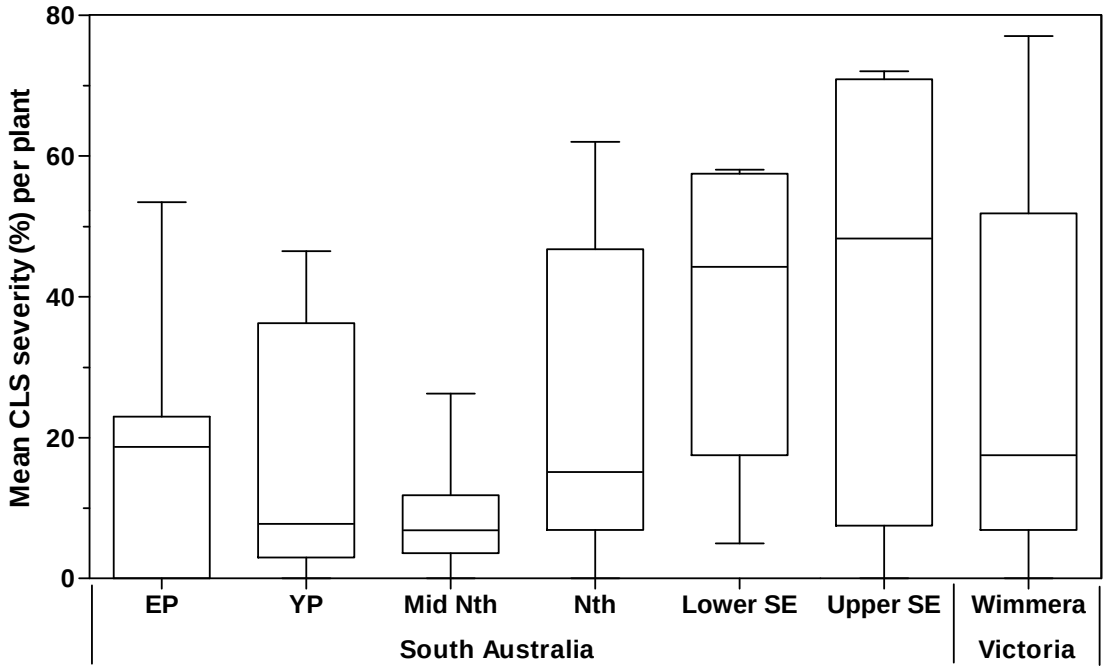

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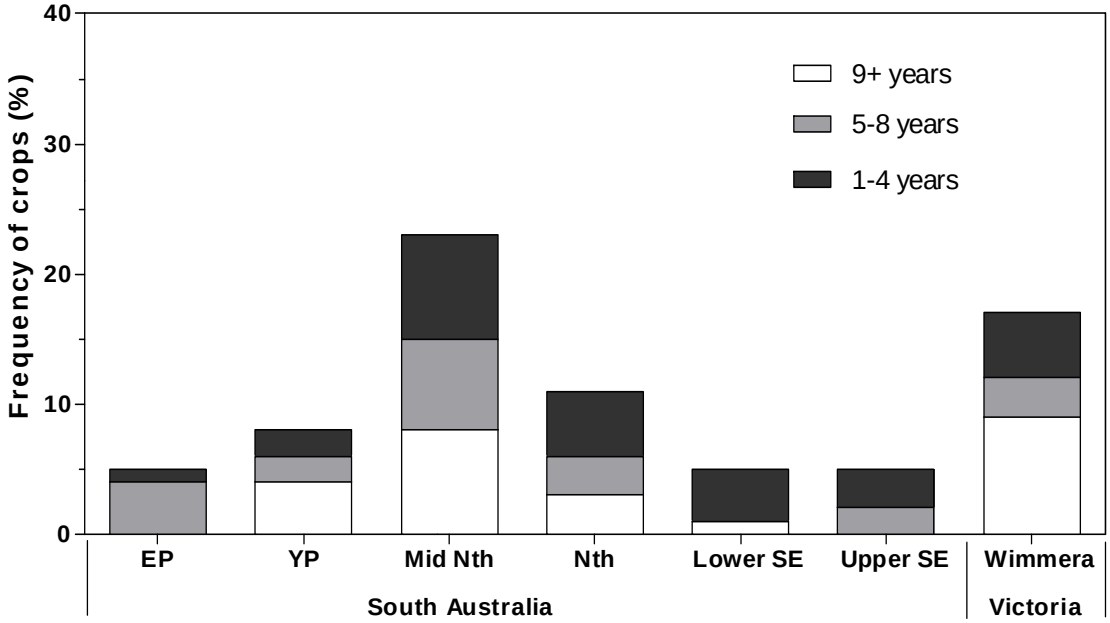

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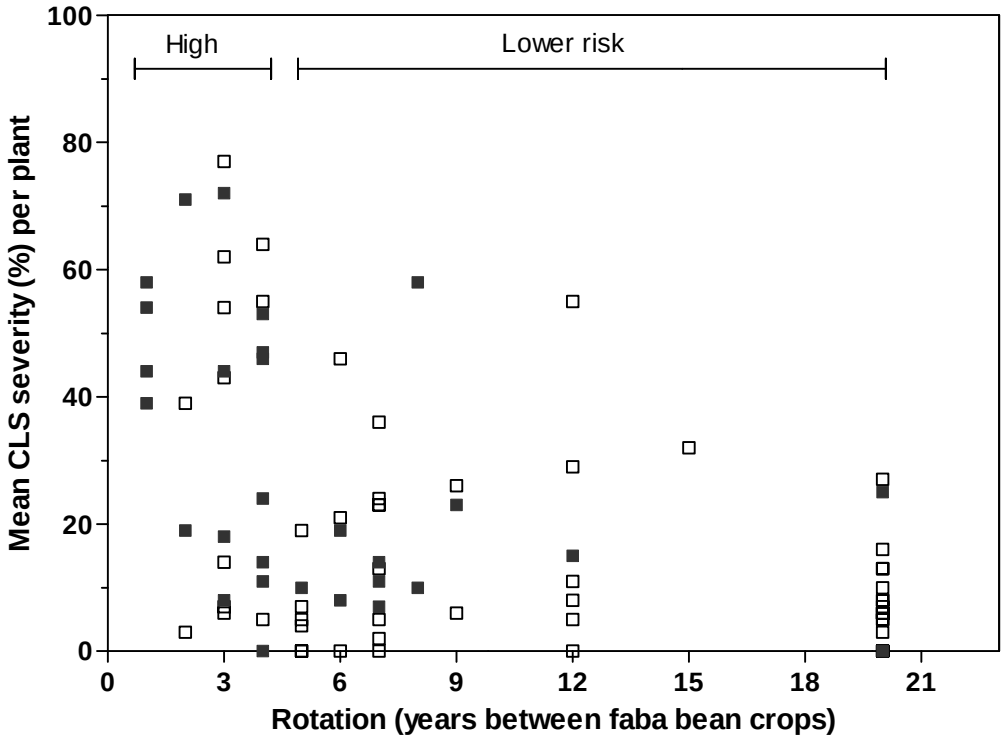

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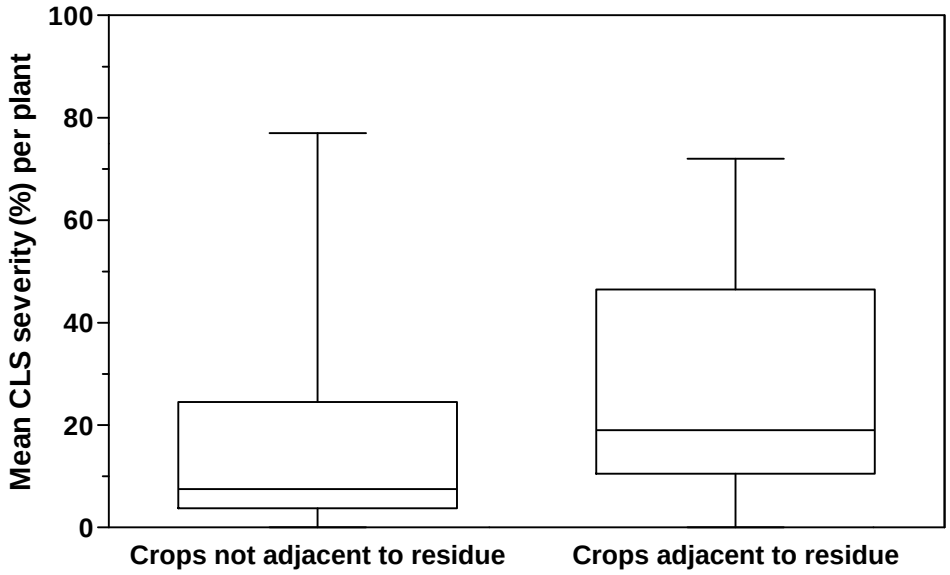

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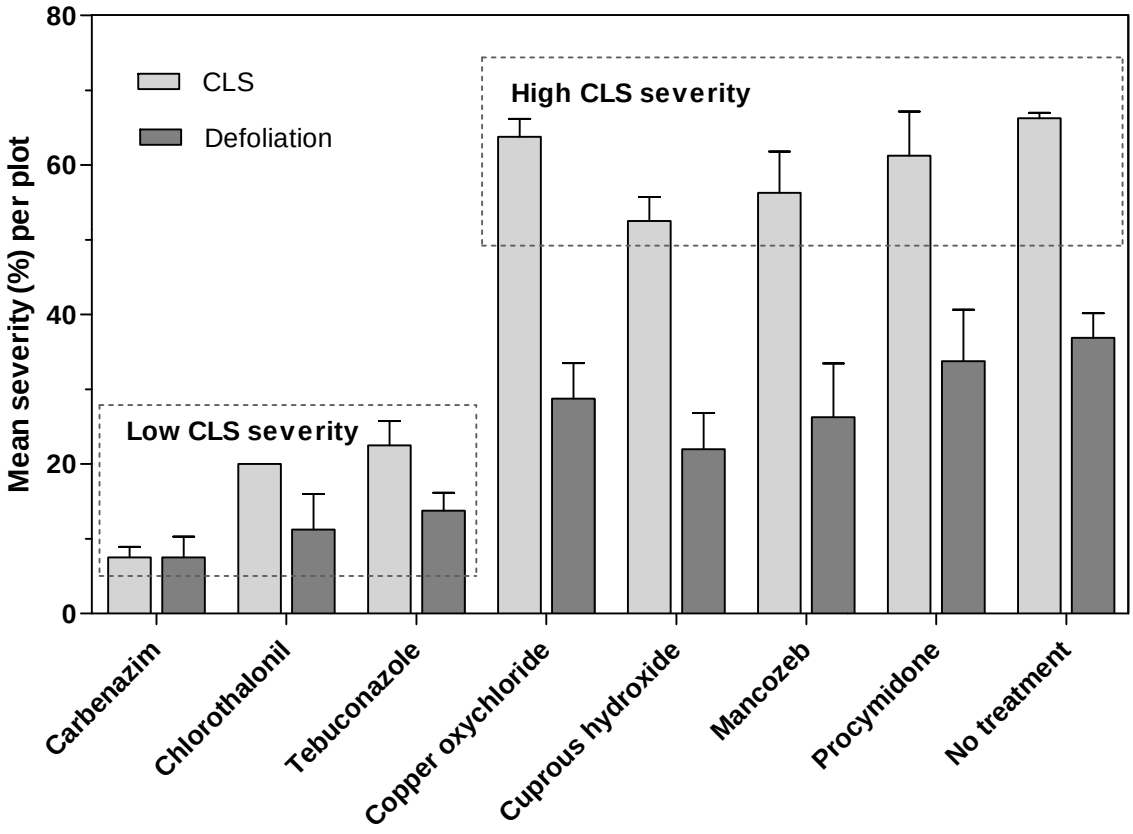

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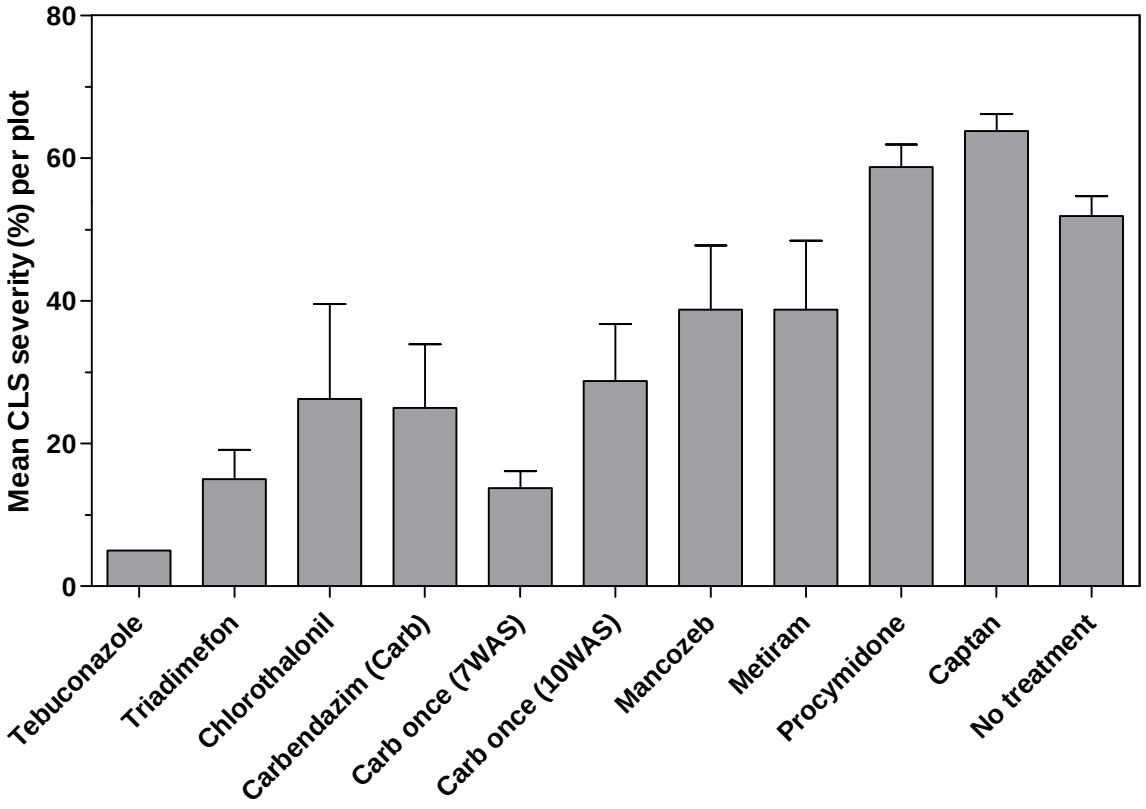

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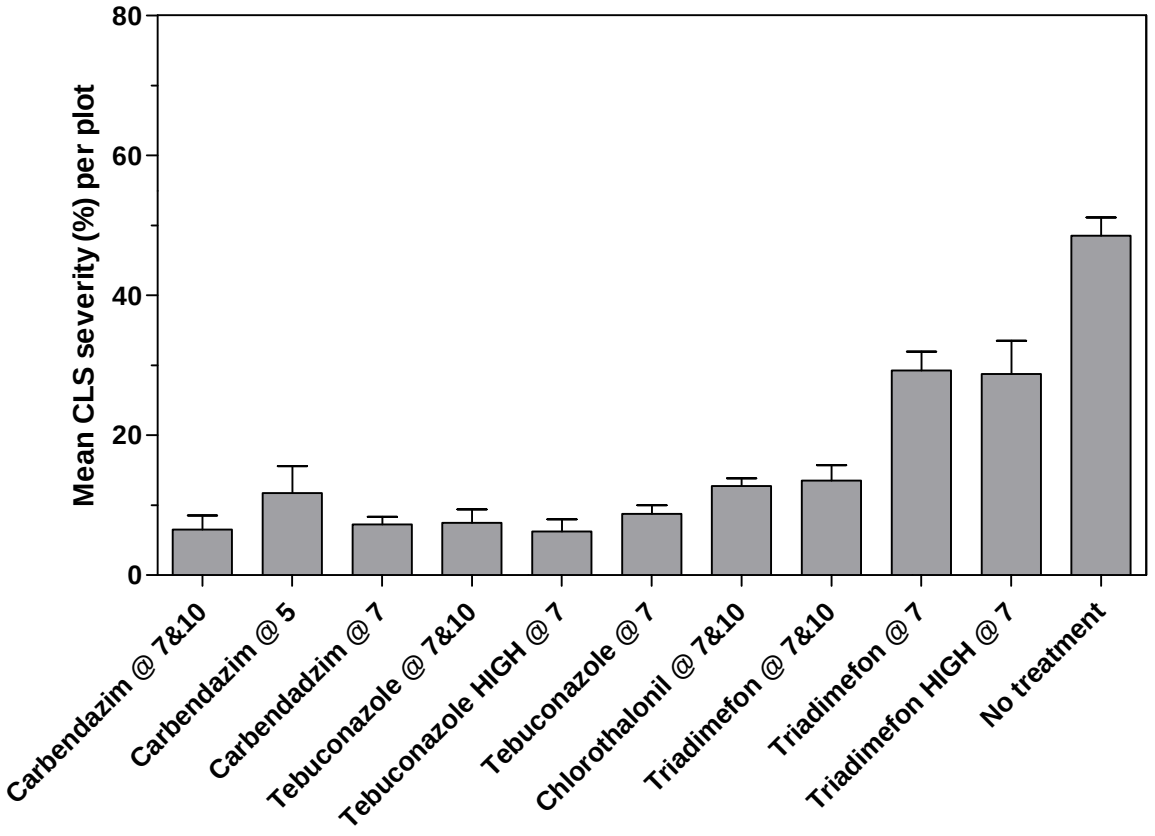

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Chapter 6.

Factors affecting infection of faba bean (*Vicia faba*) by *Cercospora zonata*

Manuscript prepared for submission to *European Journal of Plant Pathology*.

Factors affecting infection of faba bean (*Vicia faba* L.) by *Cercospora zonata*.

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Abstract. Temperature, wetness period, plant maturity, pathogen variability and inoculum concentration all influenced infection of faba bean (*Vicia faba*) by the cercospora leaf spot fungus (*Cercospora zonata*) in a controlled environment. The optimal inoculum concentration of *C. zonata* to produce disease was *ca* 10^6 mycelial fragments ml^{-1} sterilised distilled water. Symptoms included premature defoliation of the lower canopy and were influenced by plant maturity at the time of inoculation, with mid- to late-vegetative growth stages showing the most severe defoliation (40-50%). The latent period of *C. zonata* on cv. Farah declined as temperature increased and reflected the mycelial growth rate of *C. zonata in vitro* at comparable temperatures. Disease severity was positively correlated (R^2=0.83) with wet-degree hours (DH_w) and increased with temperature-weighted wetness period. Pathogenicity tests showed that 29 isolates of *C. zonata* collected throughout southern Australia from 1999 to 2008 varied in aggressiveness on faba bean. Variation could not be attributed to geographical origin of isolates or growth rate *in vitro* but analyses indicate a significant effect of time, such that a larger proportion of isolates collected in the period of 2005-2008 were aggressive than those collected in the period 1999-2004. This is the first report of pathogenic variation in *C. zonata* on faba bean and the epidemiological factors that affect disease severity.

Keywords: plant maturity, pathogen variability, epidemiology, defoliation

Introduction

Cercospora leaf spot (CLS) of faba bean (*Vicia faba* L.), caused by *Cercospora zonata*, is common in countries where faba bean is widely cultivated, however, the disease has not been examined intensively. Anecdotal evidence suggests that CLS has increased in prevalence and severity in commercial crops in southern Australia since 2004. The reasons for this are not apparent and the effect of environmental conditions on development of CLS on faba bean is poorly understood. In comparison with other *Cercospora* species, warm temperatures (22-28^{o}C) are optimal for leaf spot and blight diseases on subterranean clover, asparagus and maize caused by *C. zebrina*, *C. asparagi* and *C. zeae-maydis*, respectively (Barbetti, 1985; Cooperman & Jenkins, 1986; Paul & Munkvold, 2005) and prolonged leaf wetness is often essential for germination of conidia and infection by Cercosporoid fungi. The optimum conditions for leaf spot of peanut (caused by *C. arachidicola*) are 16 to 25^{o}C with saturated or near-saturated atmosphere, although the fungus can withstand intermittent dry periods during the infection process (Alderman & Beute, 1986; Oso, 1972; Wu *et al*., 1999). Similarly, studies of *C. beticola* on sugar beet showed that disease develops at 20 to 35^{o}C in association with leaf wetness of at least 8.5 h, but is strongly inhibited by temperatures below 10^{o}C (Windels *et al*., 1998; Wolf & Verreet, 2005). These studies indicate that prolonged wetness periods and warm temperatures promote disease development by other species of this genus and similar research may offer some explanation for the increased prevalence of CLS on faba beans in southern Australia.

Defoliation is an important symptom of many CLS diseases, reducing photosynthetic efficiency, interception of incident radiation and yield (Akem & Dashiell, 1994; Bell, 1986). Infection of peanut by *C. arachidicola* and *Cercosporidium personatum* resulted in reduced efficiency of CO_2 fixation by the remaining leaves, in addition to that attributed to defoliation, and this effect was most severe in the bottom two thirds of the canopy. Furthermore, yield was strongly correlated with amount of green leaf tissue remaining (Boote *et al*., 1980,

Subrahmanyam *et al*., 1984). Defoliation of faba bean affected by CLS has only recently been reported (Kimber & Paull, 2010) and requires further examination as no information about its effect on yield is available.

The pathogenicity of *C. zonata* to faba bean was first described by Yu (1947). While variability in this pathogenicity within *C. zonata* isolate collections has not yet been examined, inferences may be drawn from other species of the genus. Population studies of *C. zeae-maydis* have shown that variation in aggressiveness, often unrelated to the pathotypes described (designated group I and II), could in part account for variation in epidemics experienced in maize-growing districts of the US and may explain severe epidemics observed in East Africa in recent years (Wang *et al*., 1998; Carson *et al.*, 2002; Okori *et al*., 2004). Dumitras (1979) identified seven distinct races of *C. beticola* in Romania amongst *ca* 3000 isolates tested in pathogenicity studies on differential genotypes of sugar beet. Investigation of pathogenic variability of *C. zonata* may help to elucidate the factors responsible for the recent increase in prevalence and severity of CLS in commercial faba bean crops in southern Australia.

The objectives of this study were to obtain a greater understanding of *C. zonata*, and to examine factors which influence development of CLS on faba bean in controlled conditions, specifically to: quantify the effect of inoculum concentration of *C. zonata* on symptom development; elucidate the effect of plant maturity on disease severity; determine the effects of temperature and leaf wetness on disease severity following inoculation with *C. zonata*; examine the effect of temperature on growth of *C. zonata in vitro*; and examine variability in pathogenicity on faba bean and *in vitro* growth rate amongst isolates of *C. zonata* collected across southern Australia from 1999 to 2008.

Materials and methods

General methods

Since conidia of Cercosporoid fungi have not yet been produced on artificial media *in vitro,* mycelial suspensions were used in inoculations in this study (Barbetti, 1985; Booker and Umaharan, 2008; Kimber & Paull, 2010). South Australian isolate *C. zonata* 69/04, as a representative of the isolates obtained from infected faba bean leaves in 2004, was used unless otherwise stated. All isolates were stored as mycelium on plugs of potato dextrose agar (PDA; Oxoid Ltd) in sterile reverse osmosis (RO) water at 4^{o}C. Plugs were transferred to PDA in 9-cm diam Petri dishes and incubated at *ca* 22^{o}C for 2 weeks under cool-white and black fluorescent light with 12-h photoperiod. Three-week old cultures were macerated in 800 ml RO water using a commercial blender (32BL80, Waring Commercial), strained through two layers of sterile muslin cloth, then suspended in sterile RO water plus 0.05% surfactant (Tween 20; BDH Laboratory Supplies). The concentration of mycelial fragments was estimated using a haemocytometer, adjusted by dilution with RO water, and applied to seedlings using a hand-held sprayer (Hardi C5, Hardi Australia) until run-off.

V. faba cv. Farah, which is commonly grown in southern Australia and is susceptible to CLS, was used in all experiments. The CLS resistant genotype 1322/2 was used in some experiments (Kimber & Paull, 2010). Seeds were sown in pots of varying size at a depth of 3 cm in University of California potting mix (UC) (Baker, 1957).

Unless stated otherwise, the effects of treatments on disease severity were analysed using a general linear model – Analysis of Variance (ANOVA) performed in Genstat® version 11 for Windows®, data were transformed (square root) where required, and significance determined at $P<0.05$.

Effect of inoculum concentration

This experiment was arranged as a split-plot design, with the genotypes, cv. Farah (susceptible) and accession 1322/2 (resistant) as sub-plots, with three replicates. Eighteen trays, each with 12 punnets (550 ml potting mix per punnet), contained six plants of each genotype sown in the punnets on the left or right of each tray. Plants were maintained at 15^{o}C within a controlled environment room (CER) with 12-h photoperiod using eight halide-incandescent lights (400W, Osram) until four fully-expanded pairs of leaves, and two unfolded-nodes, were present. Mycelial suspensions of 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 hyphal fragments ml^{-1} RO water were dispensed into sterile McCartney bottles, 25 ml into each of three bottles per concentration. Each tray was inoculated with 25 ml of the appropriate mycelial suspension, forming the main-plots, using a hand-operated sprayer. Plants were then placed within polypropylene tents fitted with ultrasonic humidifiers (KT-100A, Humidaire) filled with RO water to maintain high humidity for 14 days, after which the humidifiers were switched off and tents opened to promote air-flow. Disease was assessed 21 days after inoculation (DAI) using a percentage (%) scale for severity (Kimber & Paull, 2010) and included; percentage leaf area diseased (LAD) on second node, percentage defoliation (where diseased leaflets abscised at the junction with the petiole), the percentage of stems exhibiting small lesions (approximately 10 mm in length) and total CLS severity (%).

Effect of plant maturity

Six pots (180 mm diameter) were filled with potting mix and each sown with four seeds of cv. Farah. This was repeated each week for 6 weeks and plants were maintained in a CER at 15^{o}C day and 10^{o}C night with 12-h photoperiod as described above. Eleven weeks after the first seeds were sown, the six growth stages (GS) were classified according to Knott (1990), comprising; late and early reproductive stages GS203(5) and GS203(1), late, mid- and early vegetative stages GS107, GS104 and GS102, and seedling stage GS004. The experiment was

arranged as a randomised complete block design with three replicates; uninoculated controls plants for each growth stage were separated by a partition to prevent cross-contamination. Inoculum was prepared as previously described, using a mixed-isolate suspension of *ca* $1x10^6$ mycelial fragments ml^{-1}, comprising eight *C. zonata* isolates (70/04 (Lower North), 73/04 and 74/04 (South East), and five listed in Table 1) collected in 2004 from different geographical locations in South Australia. Inoculum was applied to 12 plants (three pots) at each growth stage and 12 uninoculated control plants (three pots) were sprayed with RO water plus surfactant only. Immediately after inoculation plants were placed in polypropylene tents to maintain high humidity, as described previously. After 10 days, the humidifiers were switched off and the tents opened to allow air-flow. Plants were assessed 28 DAI for; percentage LAD, percent defoliation and the percentage of stems exhibiting lesions (incidence). The effect of CLS on defoliation at each growth stage was analysed by using defoliation data observed on uninoculated plants as a covariate in the ANOVA, to account for natural loss of leaves at late maturity.

Effect of temperature on growth of *C. zonata in vitro*

The growth rate of *C. zonata* isolate 69/04 in culture was examined, to compare with results for effect of temperature and wetness period on CLS severity. A single 3-mm plug extracted from the growing edge of a 14-day old culture was placed in the centre of each of 20 90-mm Petri plates of PDA. Four plates were then incubated at each of 7, 11, 15, 20 or 25^oC in one of five incubators under cool white fluorescent lighting (30W, NEC) with 12-h photoperiod. The colony diameter was measured along two perpendicular transects every 3 days for 30 days. The mean of the two transects was used to calculate growth rate over time at each temperature and data were subjected to regression analysis.

Effect of temperature and wetness period

Disease development at day/night temperature regimes of 7/4, 11/9, 15/13, 20/17 and 25/22^{o}C in combination with wetness periods of 3, 9, 24, 48, 72 and 96 h post-inoculation was examined in a series of repeated experiments using a controlled environment chamber (Environ Air) fitted with cool white fluorescent tubes (115W-F48T12, VHO Sylvania) and metal halide bulbs (150W/C/U, Venture HIE) with 12-h photoperiod. Only one chamber was available for this study and the overall experiment was arranged as a split-plot design with temperature as main plots and wetness period as sub-plots. Each temperature was repeated once, in random order, forming two replicates. Individual temperature and wetness period experiments comprised 28 pots (140 mm diameter) filled with UC potting mix and sown with cv. Farah, thinned to three seedlings per pot. The plants were initially grown in a glasshouse maintained at *ca* 20^{o}C. When seedlings had four fully-expanded leaves they were inoculated with *C. zonata* isolate 69/04 as a suspension of 1x10^{6} mycelial fragments ml^{-1}. Wetness periods were imposed by placing inoculated plants in a sealed plastic cylinder which was removed at each wetness period interval of 3, 9, 12, 48, 72 or 96 h. Four pots were subjected to each wetness period and arranged randomly in trays within the chamber. The control plants were sprayed with RO water (plus surfactant) and left uncovered. Water was poured into the bottom of the trays to keep the potting mix moist throughout the experiment.

Disease was assessed 21-60 DAI, depending on temperature, as the number of CLS lesions (per leaf-pair), the number of leaves exhibiting CLS symptoms as a proportion of all leaves and the percentage of defoliation. The mean number of lesions that developed after different wetness periods was graphed separately for each temperature and described using polynomial curves. Linear regression analysis was used to examine the relationship between CLS severity ($\sum$ mean number of leaf-nodes with lesions + mean number of leaf-nodes defoliated) and wet degree-hours (DH_w = mean temperature*wetness period), the variable used to describe temperature by wetness period.

Pathogenic variation in *C. zonata* isolates

Two experiments were conducted consecutively in a glasshouse to examine variation in pathogenicity to cv. Farah amongst 29 isolates of *C. zonata*. One isolate of *C. zebrina* (isolate 112/05) collected from infected medic (*Medicago* sp.), which is not known to be pathogenic to faba bean, was included as a negative control for pathogenicity. The isolates used in the study were collected between 1999 and 2008 from infected faba bean leaves sampled from commercial crops within the major faba bean growing regions of South Australia and Victoria (Table 1).

Each experiment consisted of 18 isolates (Table 1) in a completely randomised block design with four replicates. From mean data distribution observed in the first experiment, three categories of aggressiveness were described; low = <20% LAD, mid = 20-40% LAD and high = >40% LAD. Two isolates representing each category, selected from the first experiment, were included in the second experiment, *viz*.; 79/00 and 157/02 (low), 69/04 and YP7 (mid), VIC11 and 79/08 (high). Each experiment comprised 72 trays, each containing 12 punnets (550 ml per punnet) of UC potting mix sown with one seed of cv. Farah per punnet. Plants were grown in a natural environment that was isolated from external inoculum sources for 4 weeks (mean min/max temperatures: Experiment 1 = 15/25^{o}C; Experiment 2 = 10/20^{o}C), by which time each seedling had four leaf-nodes. Each test isolate, prepared as a 400 ml suspension of $1x10^{6}$ hyphal fragments ml^{-1}, was inoculated onto four trays (replicates). The spray equipment was disinfected with 4% sodium hypochlorite and RO water and rinsed three times between isolates. Immediately after inoculation, plants were placed in the glasshouse, with adjacent trays separated by acrylic partitions, and maintained at 18^{o}C (±4^{o}C) in high humidity (>80% RH). Disease on individual plants was assessed 14 and 18 DAI in the first and second experiment, respectively, as percentage LAD and defoliation. In addition, consecutive assessments of disease were made on each tray at 10, 12 and 14 DAI in the second experiment. Disease severity data were subjected to ANOVA as previously

described. Data were subjected to linear regression, chi-squared (χ^2) and t-tests performed using GraphPad Prism® version 5 and significance was determined at $P<0.05$. A correlation analysis was used to compare results obtained for the isolates common to the two experiments. The effect of time (year in which isolate was obtained), geography (growing region from which isolate was collected) and 'collection period' on aggressiveness was examined using chi-squared (χ^2) and t-test analysis; *C. zebrina* isolate 112/05 was excluded from this analysis. For "collection period" isolates were separated into those collected before 2005 and those collected from 2005 to 2008; the latter reflected the timing of increased prevalence of CLS in crops in southern Australia crops.

Growth rate of *C. zonata* isolates *in vitro*

The growth rate of 29 isolates of *C. zonata* and one of *C. zebrina* (Table 1) was examined *in vitro* using a completely randomised block design with three replicates (plates). Plates were prepared for each isolate by placing a single 3 mm plug from the growing edge of a 14-day-old culture onto the centre of three Petri dishes (90 mm) of PDA. These were incubated at 20°C under cool white fluorescent lighting (30W, NEC) with 12-h photoperiod in a Clayson® germination incubator. The temperature setting was chosen as the optimum temperature likely in environments during infection. The colony diameter was measured along two perpendicular transects every 3 days for 24 days and the daily mean was used to calculate the growth rate per day for each isolate. Data were subjected to ANOVA ($P<0.05$). The correlation between *in vitro* growth rate of the 29 isolates and CLS severity in pathogenicity experiments, and the correlation between *in vitro* growth rate and collection period, were determined using Graph-pad Prism® 5.

Results

Effect of inoculum concentration

Cercospora leaf spot severity (% on whole plant) on the susceptible genotype cv. Farah differed significantly ($P<0.05$) among the five concentrations of mycelial suspensions. No disease was observed on breeding accession 1322/2, thus it was excluded from the statistical analysis and data for the susceptible genotype (cv. Farah) were analysed as a randomised block design with inoculum concentration as treatments. Each 10-fold increase in inoculum concentration from 10^2 to 10^5 mycelial fragments ml^{-1} resulted in a significant ($P<0.05$) increase in mean severity of CLS (Fig. 1). This trend was also observed for percent LAD, though differences were not significant ($P>0.05$) among the three lowest concentrations. The three greatest inoculum concentrations (10^5, 10^6 and 10^7 mycelial fragments ml^{-1}) resulted in disease severity of approximately 80%, as foliage at the upper three nodes of plants, which developed after inoculation, was not diseased.

There was a significant effect ($P<0.05$) of inoculum concentration on % defoliation and on the percentage of stems that exhibited small lesions. Defoliation of plants inoculated with concentrations 10^6 (44%) and 10^7 (54%) mycelial fragments ml^{-1} was significantly greater ($P<0.05$) than for plants inoculated with concentrations of 10^5 mycelial fragments ml^{-1} and below (Fig. 1). Likewise, the percentage of stems with lesions (>15%) on plants inoculated with concentrations of 10^5 and 10^7 mycelial fragments ml^{-1} was significantly greater ($P<0.05$) than for plants that received inoculum of less than 10^4 mycelial fragments ml^{-1}.

Effect of plant maturity

Cercospora leaf spot lesions were evident 18 DAI, irrespective of the growth stage at time of inoculation. There was no significant effect ($P>0.05$) of plant maturity on CLS expressed as percentage LAD or incidence of stems lesions. Occasional small lesions (<2% foliage

affected) were observed on controls, possibly the result of contamination from neighbouring inoculated plants, and data for controls were omitted from statistical analysis of LAD. LAD of 30-50%, and up to 18% of stems affected by lesions (data not shown), were observed on plants 18 DAI (Fig. 2).

Plants inoculated at mid-vegetative and reproductive growth stages; GS104, GS107, GS203(1) and GS203(5), exhibited significantly ($P<0.05$) more defoliation (47-65%) than seedlings inoculated at emergence or the early vegetative stage, GS004 and GS102 (8-11%), respectively. Defoliation (12 to 50%) was also exhibited in control plants at late-vegetative and reproductive stages; the covariate analysis showed that this did not affect treatments. Plants inoculated at the vegetative stages of GS104 (mid) and GS107 (late) exhibited 46% and 52% defoliation, respectively. Hence defoliation due to CLS was significantly ($P<0.05$) more severe on plants inoculated at mid- and late-vegetative growth stages than at seedling (GS004 and GS102) or the late reproductive (GS203(5)) stages of development, both of which exhibited 8-14% defoliation (Fig. 2).

Effect of temperature on growth of *C. zonata in vitro*

There was a significant positive relationship ($R^2=0.94$, $P<0.001$) between temperature and *in vitro* growth rate of *C. zonata* (mm/day) (data not shown). The regression slope was y (growth rate) = 0.06x (temperature) + 0.05, although the constant was not significantly different from zero ($P>0.05$), indicating that the slope passed through the origin.

Effect of temperature and wetness period

The latent period of *C. zonata* decreased as temperature increased except for the two highest temperature settings. Necrotic lesions, 1-5 mm in diameter, were first observed on leaves 31 DAI on plants incubated at 7/4^{o}C day/night, 20 DAI at 11/9^{o}C, 14 DAI at 15/13^{o}C and 11 DAI at 20/17^{o}C and 25/22^{o}C. Lesions expanded rapidly (5-25 mm) at the higher temperatures

(≥15°C), often coalescing. Grey-white conidiophores protruded from the centre of larger lesions but were not observed on small lesions (<8 mm). All disease data was assessed 21 DAI for plants exposed to the 15/13°C, 20/17°C and 25/22°C day/night temperature regimes, 40 DAI for 11/9°C and 61 DAI for 7/4°C. There was a significant interaction ($P<0.05$) of temperature and wetness period on severity of CLS, including defoliation (Fig. 3). Defoliation was most severe (12-39%) for plants incubated at 20/17°C and 25/22°C and when wetness periods following inoculation were 48 h or longer. Wetness periods and temperature below these thresholds resulted in little defoliation and inoculated plants did not differ from controls.

Disease severity, in terms of the mean number of leaf lesions, at each wetness period and temperature fitted ($R^2>0.99$) polynomial disease curves (Fig. 4). Thus at warmer temperatures of 20/17°C and 25/22°C, disease was most severe following a leaf wetness period of 72 h. However, moderate disease (>10 mean no. of lesions plant^{-1}) was observed at cooler temperatures (9-17°C) when leaf wetness periods were 72 h or greater (Fig. 4). When disease data were combined for the total number of leaves affected by CLS and number of leaves that abscised due to CLS, severity exhibited a strong positive linear relationship ($R^2=0.83$; $P<0.001$) with DH_w (wet degree-hours) (Fig. 5). At the greatest DH_w disease was observed on six leaf-nodes, indicating that unfolded leaves present at the time of inoculation also became infected. If defoliation data were omitted from analysis of severity data, a strong positive linear relationship ($R^2=0.72$; $P<0.001$) remained between mean total number of CLS lesions on leaves and DH_w.

Pathogenic variation amongst *C. zonata* isolates

The 29 isolates of *C. zonata* examined in the glasshouse differed significantly ($P<0.05$) in pathogenicity to faba bean cv. Farah (Table 1). Disease severity at 18 DAI, in terms of LAD and defoliation, ranged from 4 to 80% and 0 to 40% defoliation of the lower canopy of

inoculated plants, respectively (Fig. 6). Disease assessments at 10, 12, 14 and 18 DAI in the second experiment showed that CLS severity increased with time for all isolates and final CLS severity varied from 6% for the least aggressive isolate to 67% for the most aggressive isolate. Plants inoculated with *C. zebrina* isolate 112/05 (designated controls), exhibited 5% LAD and 30% defoliation. This was not significantly (P>0.05) different from the least aggressive isolates of *C. zonata* (79/00, 157/02, 111/03, 77/04, 74/06). An unpaired t-test analysis of experiment 1 (grand mean 32.89 ± 5.387, N=18) and experiment 2 (grand mean 32.57 ± 4.072, N=18) showed that there was no significant difference between mean disease scores (t=0.047, df=34; P=0.96), nor between variances (F-test, P=0.26) for the two experiments.

Disease severity data for the six isolates common to both experiments were subjected to correlation analysis. This was not statistically significant (correlation coefficient R=0.37); one isolate (VIC11) caused 60% mean LAD on plants in experiment 1 and 23% mean LAD on plants in experiment 2. When this outlier was excluded, analysis of the remaining data showed a highly significant correlation (R=0.75, P=0.001) between pathogenicity of isolates common to both glasshouse experiments.

Pooled data categorised into low, mid and high disease scores showed that variation in aggressiveness was not correlated with geographical origin of the pathogen (Table 1). As much variation was observed amongst isolates collected within one faba bean growing region as amongst 29 isolates collected across six regions in southern Australia. However, variation could be partially attributed to the 'collection period' in which isolates were obtained (Fig. 7). Of the *C. zonata* isolates collected from 1999 to 2005, 55% (6 of 11) resulted in slight CLS on faba bean (mean CLS = 25.5% ± 5.4, N=11), compared with 11% (2 of 18) of isolates collected in 2005 to 2008 (mean CLS = 42.0 ± 4.3, N=18). The contrast in aggressiveness of isolates between the two collection periods was significant in contingency tests applied to

pathogenicity categories ($\chi^2 = 8.6$, P=0.01) and in an unpaired t-test analysis of the mean CLS severity caused by isolates in each collection period (t=2.37, P<0.05, df=27).

Growth rate of *C. zonata* isolates *in vitro*

There were significant (P<0.05) differences among the growth rates of the 29 isolates of *C. zonata* examined *in vitro* (Table 1). Growth rates ranged between 0.86 and 1.41 mm day^{-1}, which was significantly (P<0.05) less than that for *C. zebrina* isolate 112/05 (1.67 mm day^{-1}). However, there was no significant relationship between growth rate of *C. zonata* isolates and severity of CLS when inoculated on plants (R^2=0.03, P=0.38). Additionally, variation in growth rate could not be attributed to the year in which the isolate was collected (chronology) or geographical origin.

Discussion

The severity of CLS was influenced by inoculum concentration, plant maturity, temperature, wetness period and isolate of *C. zonata.* The level of disease on the susceptible faba bean cv. Farah increased with increasing concentration of mycelial fragments in the inoculum suspension. Suspensions of $\geq 10^5$ mycelial fragments ml^{-1} resulted in severe CLS, in terms of LAD (>40%), defoliation (>25%) and stem lesions (>14%), and defoliation was more severe on plants inoculated with 10^6 and 10^7 mycelial fragments ml^{-1} than lesser concentrations. As defoliation is an important component of CLS (Kimber & Paull, 2010), 10^6 mycelial fragments ml^{-1} was selected as the minimum inoculum concentration required for subsequent experiments to examine factors affecting disease severity. Mycelial suspensions, likewise, have served as an alternative to conidiospore inoculum for a number of *Cercospora* spp. that fail to sporulate in culture (Barbetti, 1985; Schuh, 1992; Tessman *et al*., 2008). Though further studies that examine spore- versus mycelia-induced infection are recommended, this

technique was used to evaluate resistance to CLS in faba bean in controlled environments and showed a strong correlation with field tests with natural infection (Kimber & Paull, 2010).

Premature defoliation of lower leaves occurred on faba bean plants infected with *C. zonata*. The onset of defoliation was influenced by plant maturity at the time of inoculation, such that 40-50% of lower leaves abscised when inoculated at mid-vegetative growth stages whereas only 2-10% of lower leaves on control plants abscised at the same growth stages. However, plant maturity at the time of inoculation did not appear to influence LAD, which was severe in all treatments. Healthy leaf duration and leaf area absorption are often used to predict both biological yield (i.e. photosynthesis, nitrogenase activity) and seed yield of numerous plants (Waggoner & Berger, 1987; Lopes & Berger, 2001) and the rate of leaf production and unfolding in faba bean reaches a critical point at early flowering after which leaf production responds to stress (Karamanos, 1978). The premature loss of lower leaves induced by CLS observed in this study occurred at this critical point of plant development. Combined with effects of leaf area diseased, this it is likely to reduce the capacity for photosynthesis and the efficiency with which the intercepted radiation is utilised by affected plants (Lopes & Berger, 2001). Grain yield loss in faba bean due to CLS, manifesting as severe LAD and defoliation, was first identified in field trials conducted in 2005 in South Australia (unpublished data). Mechanical defoliation (50-100%) decreased yield of field-grown sunflower and soybean, particularly when imposed at vegetative or early-flowering growth stages than later growth stages (Muro *et al*. 2001; Proulx & Naeve 2009). However, less severe defoliation treatments (33%) resulted in negligible impact on yield, which indicated a quantitative threshold for reduced leaf area before yield is compromised (Muro *et al*. 2001). This emphasises a need for further research to quantify the effects of CLS on leaf area duration, interception of light and yield in faba bean.

The decrease in latent period as temperature increased suggested that temperature is important in the infection of faba bean by *C. zonata*. Similar findings were reported after

controlled environment studies of other plants infected by other *Cercospora* species (Wadia & Butler, 1994; Inman *et al*., 1997). However, the shortest latent period for *C. zonata* in our studies was 11 days at 25°C, in contrast to 2-3 days reported by Yu (1947), though incubation temperatures in that study were not specified. Our study also demonstrated a positive correlation between temperature and growth rate of the same isolate of *C. zonata in vitro*, supporting the assumption that the reciprocal of the latent period is often a measure of the rate of pathogen development (Wadia & Butler, 1994). However, at higher temperatures (>25°C), which were not examined in this study, this relationship is expected to be non-linear (Wadia & Butler, 1994).

Warmth was favourable for CLS, with the greatest disease intensity observed at the highest temperature regime tested (25/22°C, day/night). The influence of temperature on disease severity, however, depended on leaf wetness period. The combined effects of temperature and wetness period on the mean number of CLS lesions were described here using polynomial equations (R^2>0.97) and identified an apparent optimal wetness period of 72 h at 20-25°C. Similar relationships have been reported for a variety of diseases (Elliot, 1988; Arny & Rowe, 1991; Butler *et al*., 1994). However, the total number of leaf nodes affected by CLS was considered to be a more appropriate response variable for disease severity because it represented both leaf loss and leaves with lesions. The interactive effect of temperature and wetness period on CLS severity was then simplified using calculated wet degree hours (DH_w) to combine thermal time units (degree hours) accumulated during wetness periods. This approach has been used previously to describe pathogen phenology and does not attribute upper infection limits to either temperature or wetness duration (Pfender, 2003). A regression analysis showed that CLS severity exhibited a strong positive relationship with DH_w and this accounted for over 80% of the variance in our data. Likewise, studies on other *Cercospora* species have shown approximately 25°C, combined with a long leaf wetness period, to be optimal for disease (Shew *et al*., 1988, Wadia & Butler, 1994).

Temperatures above 25°C were not examined however, so the upper threshold temperature for CLS was not determined. Furthermore, *C. zonata* also infected faba bean at cooler temperatures (7-15°C) after long periods of leaf wetness, which would allow early establishment of the disease in the field during winter. The complex relationship between temperature, leaf wetness and fluctuations of either parameter could be examined in detail at temperatures below 20°C, which are experienced in southern Australia during early crop development when the disease is first observed. Additionally, relationships that examine spore- versus mycelia-induced infection are required, since conidia are an important source of inoculum within natural environments for this disease. However, *C. zonata* mycelia were used to evaluate resistance to CLS in faba bean in controlled environments and showed a strong correlation with field trials originating from natural infection (Kimber & Paull, 2010).

C. zonata isolates collected within southern Australia between 1999 and 2008 showed significant variation in aggressiveness when tested on a susceptible faba bean genotype in glasshouse experiments. This is the first report of pathogenic variability in *C. zonata* on faba bean. Bair & Ayers (1986) reported a similar finding amongst 15 isolates of *C. zeae-maydis*, and that variation in aggressiveness may reflect pathogen fitness. That study showed a group of isolates originating from one growing district exhibited reduced fitness compared to other isolates examined, although it was concluded that variation across all isolates was not associated with location from which they were collected. Our analysis of pooled pathogenicity data showed that variation in aggressiveness in *C. zonata* isolates could not be attributed to the geographical origin of the pathogen. In fact, there was as much variation amongst isolates collected within one growing region as amongst 29 isolates collected across southern Australia. Furthermore, aggressiveness was not related to the growth rate of the fungus in culture, as determined in a regression analysis. However, when isolates were grouped into two collection periods, there was evidence that variation in aggressiveness might be attributed to year of collection. Aggressive isolates were more common among those

collected in 2005-2008 than in the 1999-2004, and this is reflected in the increase in the prevalence and severity of cercospora leaf spot in commercial faba bean crops in southern Australia after 2004. However, the most aggressive isolate had been collected from the south east of South Australia in 2000 and stored in water at 4^{o}C for 8 years before testing. This suggests that pathogenicity had not diminished as a result of storage. Although differences in aggressiveness might be attributable to effects of host cultivar (Caten, 1974), a factor not investigated in this study, there is no evidence that the susceptibility of commercial cultivars of faba bean to CLS has increased during 1999-2008 (Kimber & Paull, 2010). However, the collections of *C. zonata* isolates available that represented each time period and geographical origin were not balanced in this study, as the collection of isolates was proportional to disease incidence in the field and priorities in disease surveillance at the time. As a result, 18 isolates obtained during 2005 to 2008 were compared with only 11 isolates collected during 1999 to 2004 and this may have contributed to a greater number of aggressive isolates being identified in the later time period.

This study of factors that influence the development of CLS on faba bean suggested that plant growth stage at the time of inoculation with *C. zonata* can influence disease severity, particularly the onset of defoliation. Furthermore, severity of CLS was positively correlated with temperature-weighted wetness period, and was greatest at 15 to 25^{o}C when wetness periods were 72 to 96 h. In south-eastern Australia, average maximum temperatures have increased during the latter half of the 20th century (Nicholls, 2004) and more specifically, from 2002 to 2009 the average maximum temperatures for the spring and early summer months of September to December was 0.7^{o}C to 2.2^{o}C higher at the field sites used in this study compared to the long term average (1889 to 2009) (BOM, 2007; McMurray *et al*., 2011). This may have contributed to the complex interactions that resulted in the increased prevalence and severity of CLS in southern Australia since 2004. Further research is recommended to examine *C. zonata* populations for shifts in pathogenicity, or the existence of

pathotypes or races. Detailed field studies are also warranted to examine the influence of climate and inoculum source on faba bean genotypes differing in resistance to CLS, to understand the potential impact on the southern Australia industry if temperatures continue to increase.

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Table 1 Description of 29 *Cercospora zonata* isolates, and one *Cercospora zebrina* isolate (112/05) from medic (*Medicago* sp.) used as a control, tested for pathogenicity to faba bean (*Vicia faba*). Isolates were collected from major faba bean growing regions in South Australia (SA) and Victoria (VIC) from 1999 to 2008. Cercospora leaf spot (CLS) severity (mean %) for each isolate observed in pathogenicity tests conducted in the glasshouse, and category of aggressiveness assigned, are presented

Year	Accession number	State	Growing Region	Growth rate *in vitro* (mm day^{-1})[a]	CLS (%)[b]	Aggressive category[c]
1999	122/99[d]	SA	South East	0.99	38.2	mid
2000	79/00[d,e]	SA	South East	0.91	7.0	low
2000	80/00[e]	SA	South East	1.18	66.7	high
2002	157/02[d,e]	SA	South East	1.25	3.4	low
2003	111/03[d]	SA	Lower North	1.20	7.8	low
2003	131/03[e]	SA	South East	1.41	6.3	low
2004	37/04[e,f]	SA	Mid North	1.20	12.3	low
2004	69/04[d,e,f]	SA	Lower North	1.17	26.0	mid
2004	71/04[e,f]	VIC	Wimmera	0.86	39.4	mid
2004	72/04[d,f]	VIC	Wimmera	1.07	29.2	mid
2004	77/04[d,f]	SA	Mid North	1.11	11.6	low
2005	48/05[e]	SA	Lower North	0.99	63.6	high
2005	70/05[e]	SA	Yorke Peninsula	1.23	35.6	mid
2005	112/05[d]	SA	Lower North	1.67	5.6	control
2005	180/05[d]	SA	South East	1.05	42.8	high
2006	29/06[d]	SA	Eyre Peninsula	1.04	67.3	high
2006	32/06[e]	SA	Eyre Peninsula	1.08	37.2	mid
2006	74/06[d]	SA	Eyre Peninsula	1.17	4.3	low
2007	EP3[e]	SA	Eyre Peninsula	1.07	22.3	mid
2007	PC4[e]	SA	Mid North	0.96	27.9	mid
2007	MF 10[e]	SA	Mid North	1.15	42.1	high
2007	PH6[d]	SA	Mid North	1.07	51.2	high
2007	AP2[e]	SA	Lower North	0.99	12.8	low

2007	YP7[d,e]	SA	Yorke Peninsula	1.31	23.3	high
2007	SE8[e]	SA	South East	1.12	27.5	mid
2007	SE17[d]	SA	South East	1.11	65.8	high
2007	VIC11[d,e]	VIC	Wimmera	1.13	23.1	high
2007	VIC19[d]	VIC	Wimmera	1.13	37.5	mid
2008	79/08[d,e]	SA	Mid North	0.98	52.6	high
2008	80/08[d]	SA	Mid North	1.11	58.0	high

[a] Daily mean growth rate calculated from a 3-mm culture plug placed onto potato dextrose agar and incubated at 20^{o}C under 12 h photoperiod of cool white fluorescent lighting (30W, NEC) for 24 days

[b] Mean disease severity on faba bean (cv. Farah) 18 days after inoculation with 10^6 mycelial fragments ml^{-1} and maintained at 18^{o}C (±4^{o}C) in constant high humidity in glasshouse pathogenicity tests

[c] Aggressiveness determined from glasshouse pathogenicity tests: low = ≤ 20% CLS, mid = > 20 < 40% CLS and high = ≥ 40% CLS

[d] Isolate included in experiment 1 to examine pathogenic variation

[e] Isolate included in experiment 2 to examine pathogenic variation

[f] Isolate used in experiment to examine the effect of plant maturity

Figure Legends

Figure 1 Effect of inoculum concentration on severity of cercospora leaf spot (CLS) on faba bean (cv. Farah) 21 days after inoculation with six concentrations of mycelial fragments of *Cercospora zonata* isolate 69/04. Error bars represent $LSD_{0.05}$ for each parameter used to measure disease severity: % CLS severity = total cercospora leaf spot severity; %LAD = mean % leaf area diseased

Figure 2 Effect of plant maturity on cercospora leaf spot (CLS) severity in faba bean (cv. Farah) assessed 21 days after inoculation at six plant growth stages with *Cercospora zonata* (isolate 69/04). Growth stages represent seedling (GS004), vegetative (GS102-7) and reproductive (GS203) according to Knott (1990). $LSD_{0.05}$ Leaf Area Diseased (LAD) = 8.5%, $LSD_{0.05}$ Non-disease defoliation = 20.3%, $LSD_{0.05}$ Defoliation due to CLS = 24.0%

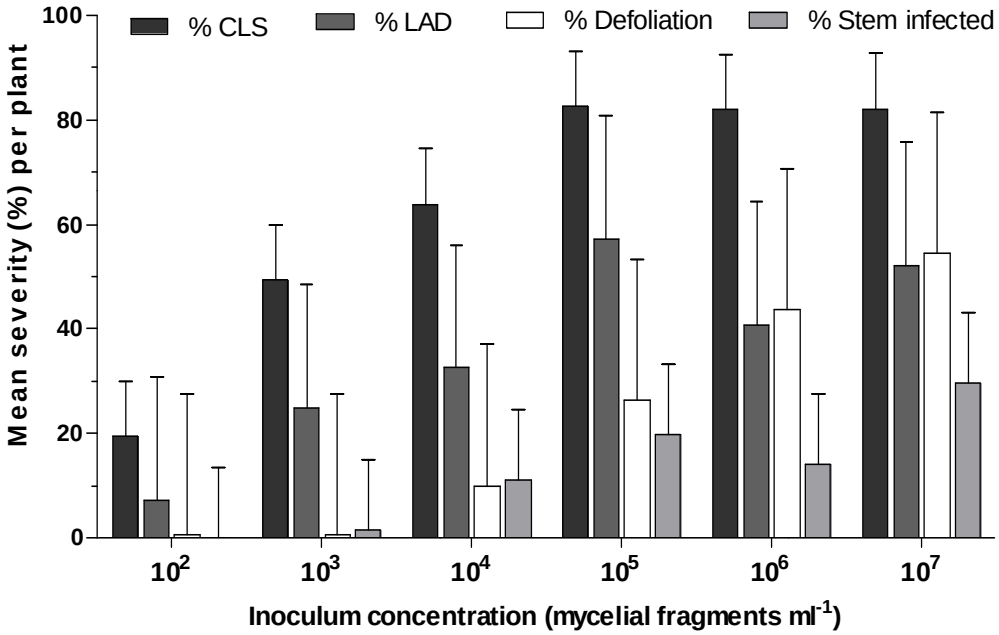
Figure 3 The effect of temperature and wetness period on % defoliation of faba bean (cv. Farah) after inoculation with mycelia of *Cercospora zonata* isolate 69/04. Control plants were inoculated with water plus wetting agent and a wetness period was not applied. Percent defoliation was assessed 21 days after inoculation (DAI) for temperatures regimes 15/13^{o}C, 20/17^{o}C and 25/22^{o}C, 40 DAI for 11/9^{o}C, and 61 DAI for 7/4^{o}C day/night. $LSD_{0.05}$ (temperature*wetness period) = 11.03

Figure 4 Relationship between wetness period (h) and severity of cercospora leaf spot (mean number of leaf lesions) on faba bean (cv. Farah) incubated at five temperature regimes (day/night). Leaf wetness periods of 3, 9, 24, 48, 72 and 96 hours were maintained after seedlings (four leaf nodes) were inoculated with *Cercospora zonata* (isolate 69/04). Lesions were counted 21 days after inoculation (DAI) at 15/13^{o}C, 20/17^{o}C and 25/22^{o}C, 40 DAI for 11/9^{o}C, and 61 DAI for 7/4^{o}C day/night. $LSD_{0.05}$ (temperature*wetness period) = 9.59. Polynomial relationships (R^2>0.99) fitted to disease data at 7/4^{o}C (●, $y=0.001x^2-0.02x+0.11$), 11/9^{o}C (■, $y=0.003x^2–0.11x+0.83$), 15/13^{o}C (△, $y=0.005x^2–0.16x+1.97$), 20/17^{o}C (□, $y=-0.00x^3+0.01x^2–0.28x+1.46$) and 25/22^{o}C (×, $y=-0.00x^3+0.02x^2–0.06x+3.48$)

Figure 5 Cercospora leaf spot severity (combined mean data = mean number of diseased leaves + mean number of defoliated leaves) on faba bean plants (cv. Farah) maintained at five day/night temperature regimes of 7/4, 11/9, 15/13, 20/17 and 25/22^{o}C and leaf wetness periods of 3, 9, 24, 48, 72 and 96 h after inoculation at four exposed leaf node stage with *Cercospora zonata* isolate 69/04. Variable DH_w = mean temperature*wetness period

Figure 6 Box and Whisker plot showing variation in pathogenicity, measured by Leaf Area Diseased (% LAD) and % defoliation on *Vicia faba* cv. Farah, amongst 18 isolates of *Cercospora zonata* in two separate glasshouse experiments (29 isolates in total). The central portion of the box represents 50% of the data, separated by the median, and whiskers extend to the lower and upper 25% of data

Figure 7 Effect of isolate collection period on severity of cercospora leaf spot (CLS) on faba bean (cv. Farah) caused by 29 isolates of *Cercospora zonata* collected from 1999 to 2008. Aggressiveness was categorised as; Low = mean Leaf Area Diseased (LAD) <20%, Medium = mean LAD 21-39%, High = mean LAD >40%. The difference between aggressiveness of isolates collected from 1999 to 2004 (mean CLS severity = 25.5% ± 5.4, N=11) compared to isolates collected from 2005 to 2008 (mean CLS severity = 42.0 ± 4.3, N=18) was significant (χ^2 = 8.6, *P*=0.01; t=2.37, *P*<0.05, df=27)



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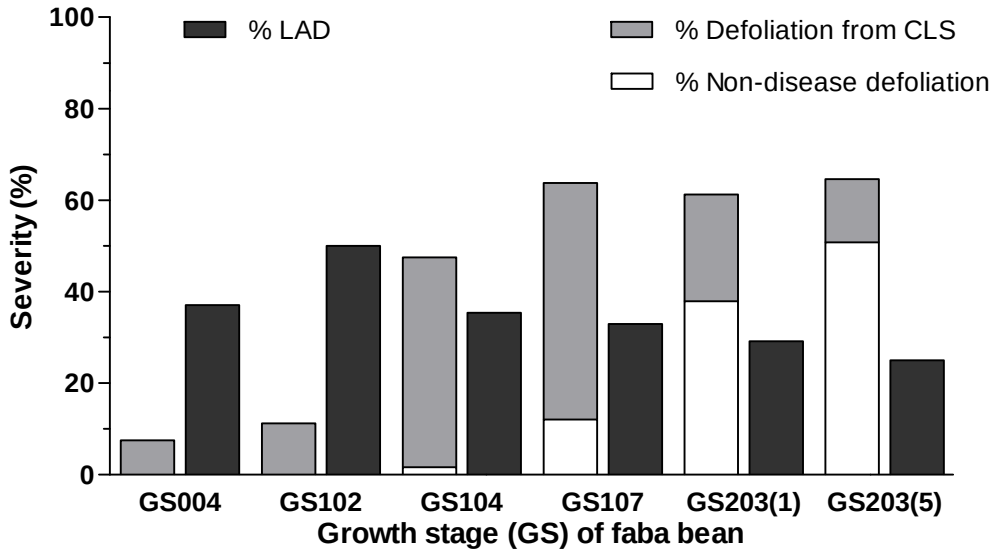

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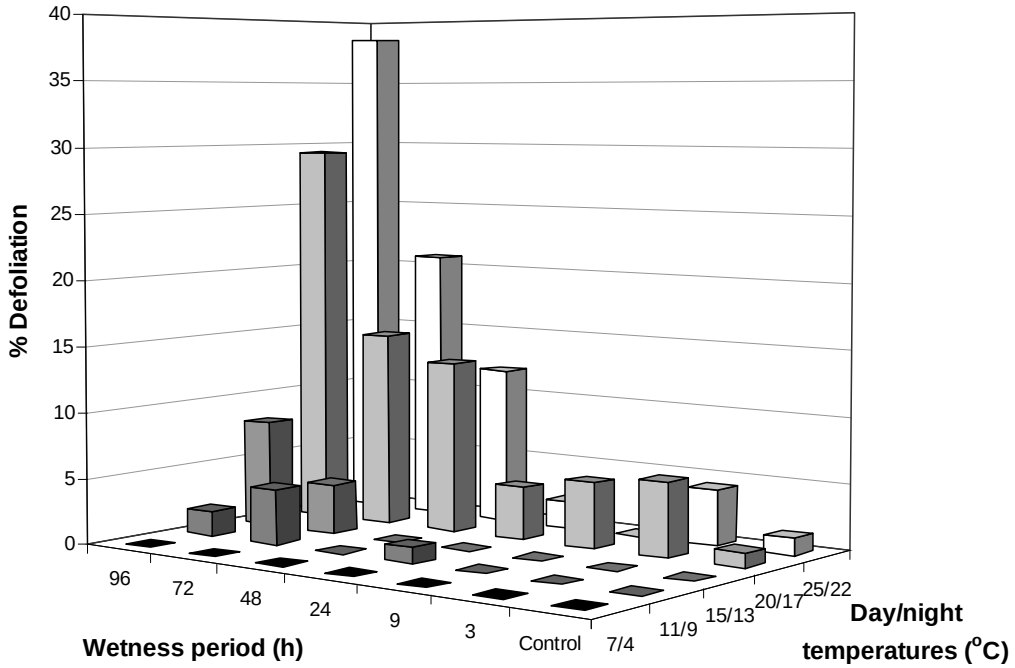

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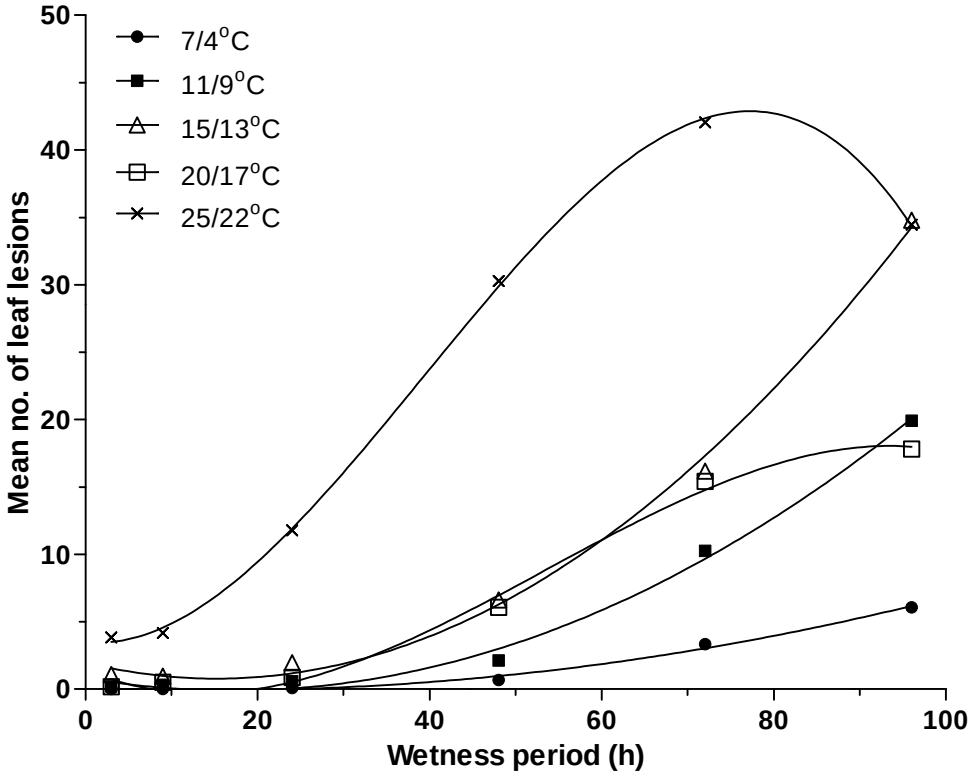

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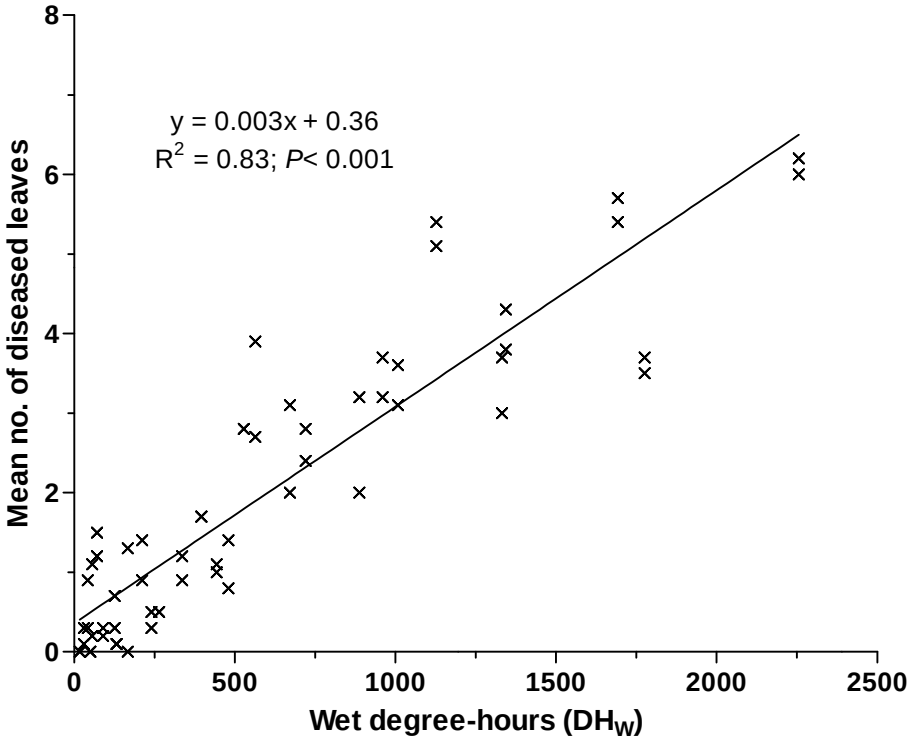

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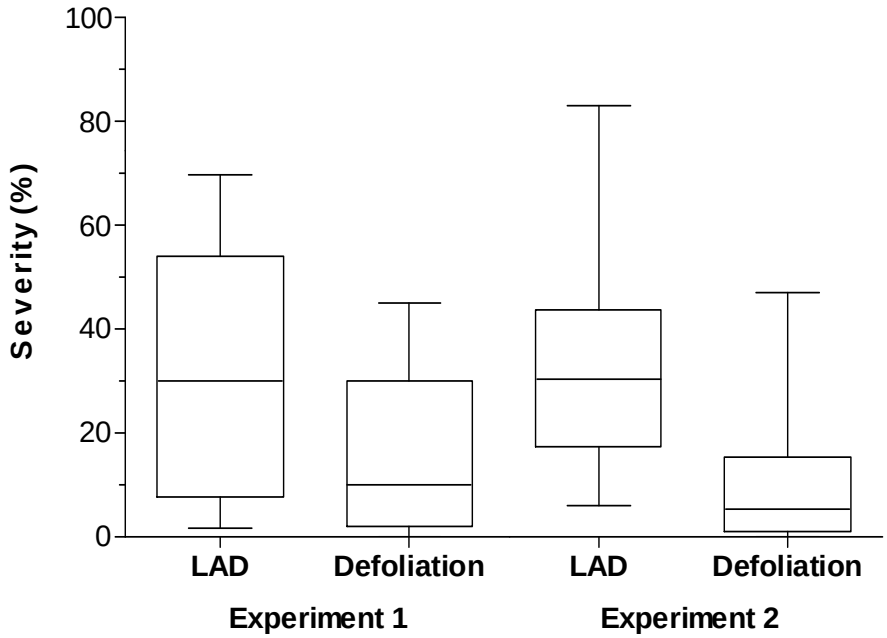

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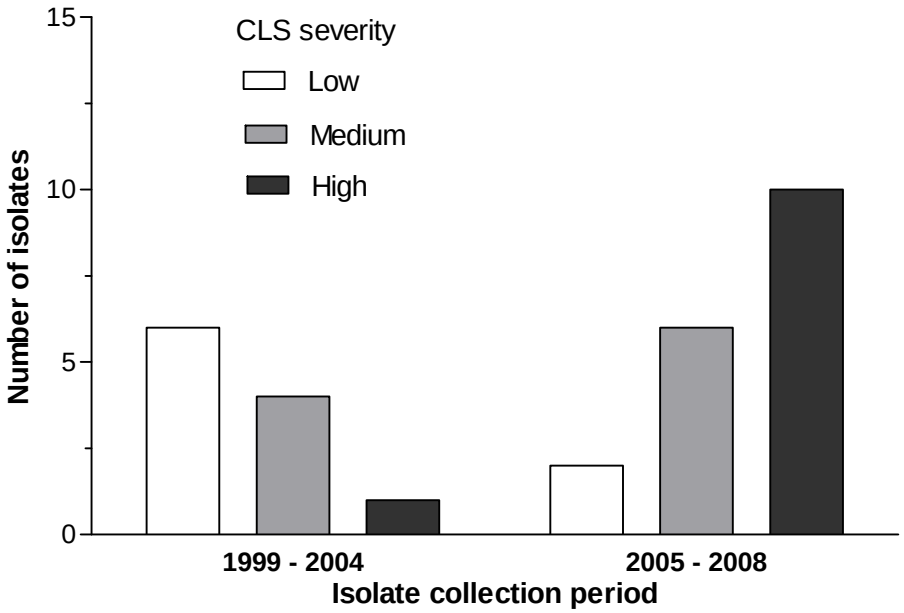

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Chapter 7.

Temporal and spatial development of cercospora leaf spot of faba bean influenced by *in situ* inoculum

Manuscript prepared for submission to *Plant Disease*, except that citations are in the form of author and date, rather than numbers, to be consistent with the format used elsewhere in this thesis.

Temporal and spatial development of cercospora leaf spot of faba bean influenced by *in situ* inoculum

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ABSTRACT

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The temporal and spatial dynamics of cercospora leaf spot (CLS) on susceptible and resistant genotypes of faba bean grown in or at defined distance from soil with infested residues was examined. Disease was first observed on susceptible seedlings 49 days after sowing (DAS) in soil that had been sown with faba bean every 3 years since 1997, but was delayed by one week in adjacent soil with no history of cultivation of faba bean. Spatial patterns of diseased

seedlings between 49 and 63 DAS showed a gradient from 4 to 16 m from the infested soil. CLS (mean leaf area diseased, %LAD) was severe (85% ± 4.3%) on the three nodes closest to the soil surface, and much less severe (1% ± 0.6%) in the upper canopy. Defoliation, which was combined with data for %LAD to describe the loss of photosynthetic leaf area (%LPLA), was an important component of CLS severity. Non-linear regressions using a logistic model described disease development over time on susceptible plants in soil with *in situ* inoculum, whereas an exponential model best described disease gradients with distance from the inoculum source and disease development on resistant plants. Paired *t*-test analyses of fitted values (%LPLA) at 77 and 98 DAS showed significant differences in disease severity in the two soil zones and a steep gradient 0 to 4 m from the inoculum source. There was a positive relationship (R^2=0.93, P<0.05) between disease severity on susceptible plants in soil where infested residue had remained on the surface and the amount of DNA of *C. zonata* detected in the soil. When residues were removed from the soil surface, or depleted rapidly through grazing, the infectivity of soil and the amount of DNA of *C. zonata* detected were significantly less than for soil with residue remaining on the surface. *C. zonata* survived in soil and remained infective for at least 30 months.

INTRODUCTION

Cercospora leaf spot (CLS), caused by *Cercospora zonata*, is a fungal disease that affects faba bean (*Vicia faba* L.) in most countries where the crop is cultivated. However, the disease is not considered a major threat to faba bean production and, consequently, few researchers have examined the life cycle of *C. zonata* (Woodward 1932; Yu 1947). There is evidence that in the Mediterranean-type environments of southern Australia, CLS has increased in prevalence and severity in commercial crops since 2004 (R. B. E. Kimber, *unpublished data*). The reasons for this increase are not obvious. In the absence of cultivars

with resistance to CLS (Kimber & Paull, 2011) disease management will rely on strategies based on knowledge of pathogen survival, dissemination and development. However, the epidemiology of CLS on faba bean is poorly understood and this study was intended to investigate some of the gaps in knowledge of survival and spread of the disease.

Conidia of most *Cercospora* species are borne on erect conidiophores formed as fascicles above the leaf surface which protrude into the turbulent air (Meredith, 1973). The conidia are passively detached and are dispersed by wind or rain-splash (Williams, 1987; Lapaire and Dunkle, 2003). *C. zonata* is presumed to persist as fascicles or as dormant stromatic mycelium in crop residue remaining on the soil surface (Yu, 1947; Walker, 1952) and the principal agents for dispersal of conidia from infested residue on the soil surface are wind and rain, which influence spatial patterns of disease distribution from the inoculum source (de Nazareno et al, 1993; Ward et al., 1999; Vereijssen et al, 2006). Reports on dispersal of *C. beticola*, the causal agent of CLS on sugar beet, show that spatial patterns are less distinct where wind is the principal dispersal agent, compared to rain-splash, since dissemination of disease may occur some distance from infected crops, such as to neighbouring fields (Lawrence & Meredith, 1970; Khan et al, 2008). In contrast, the incidence of CLS on sugar beet arising from conidia dispersed by rain-splash decreased over short distances, so that infection from infested debris at the soil surface occurred first on the lower leaves of the host, and wind played a secondary role in inoculum dispersal (McKay & Pool, 1918, Windels et al, 1998; Vereijssen et al, 2006). Such information is required for CLS on faba bean, to elucidate the factors that may influence disease incidence and spread in commercial crops in southern Australia and improve management strategies for control of the disease.

Likewise, survival of *C. zonata* has not been studied. Preliminary field studies in China demonstrated that the pathogen could survive for 8 months on diseased leaf material after shallow burial in soil (Yu, 1947). The current recommendation for Australian growers is

to maintain approximately 4 years between faba beans crops within one field, by which time infested residues with survival structures of other major pathogens (*Ascochyta fabae*, *Botrytis fabae*) have typically decomposed and no longer represent a significant source of inoculum (Wallen and Galway, 1977; Dyke and Prew, 1983). Although the persistence of CLS inoculum is not known, the recent increase in disease incidence in fields where faba bean has been planted every 3-4 years suggests that the pathogen may remain viable for this period (R. B. E. Kimber, *unpublished data*). Survival of other *Cercospora* species on infested residues varied from several months to several years when residues were retained on the soil surface, but decreased dramatically when residues were buried (Nagel, 1938; Kilpatrick, 1956; Payne & Waldron, 1983; Cooperman et al., 1986; de Nazareno et al., 1992). Consequently, cultural practices to reduce residue, such as burial by tilling the soil, are often used in management of Cercospora diseases to reduce inoculum carry-over. However, widespread adoption of minimum-tillage practices to increase organic matter and improve soil structure in Mediterranean climates, such as in southern Australia, can lead to slow decomposition of residues particularly during long periods of hot, dry conditions during the non-cropping phase over summer. As a result, retaining stubble may promote survival of plant pathogens (Summer et al., 1981; Boosalis et al., 1986) and, perhaps, has played a role in the increase of CLS in Australian faba bean crops.

Study of the survival of a pathogen on infested residues or in soil requires knowledge of the life-cycle, techniques for liberating and identifying primary inoculum, and selective culture medium, techniques which, to date, have not been developed for *C. zonata*. The Root Disease Testing Service (RDTS) at the South Australian Research and Development Institute (SARDI) has developed a DNA-based assay, involving the amplification of target DNA in the polymerase chain reaction (PCR), and offers a commercial diagnostic test for pathogens in soil and seed samples (Ophel-Keller et al., 2008). DNA primers specific to *C. zonata*, based on the internal transcribed spacer (ITS) region, developed by CSIRO Entomology in

Canberra, Australia, are used in this assay to detect the pathogen in soil. This assay offers a rapid means of detecting and quantifying inoculum of *C. zonata*.

The objectives of this study were to understand: (i) the role of soilborne inoculum in establishment of CLS on faba bean and the spatial and temporal development of the disease in the vicinity of this inoculum; (ii) differences in disease development on resistant and susceptible genotypes; and (iii) the long-term survival of *C. zonata* in the soil and the role of infested residue in providing soilborne inoculum.

MATERIALS AND METHODS

Effect of cropping history on incidence and severity of CLS – General methods. Two field trials were conducted, one in 2005 and another in 2006, at the Turretfield Research Station (Latitude: 34.55°S, Longitude: 138.83°E) located 10 km north-east of Gawler, South Australia. The region has a Mediterranean climate with mean annual rainfall 460 mm, 80% of which falls between April-October, elevation 116 m, and red-brown loam soil. Each trial was situated on a site where faba beans had been grown every 3 years, since 1997, allowing the natural establishment of CLS. The trials were arranged so that 50% of plots were on soil in which faba bean had been planted every 3 years (positive (+) soil zone) and 50% on adjacent soil with no history of faba bean (negative (-) soil zone). Two genotypes of *V. faba* were examined, one susceptible (cv. Farah) and one resistant (breeding accession 1322/2) to CLS (Kimber and Paull, 2011). Each trial consisted of 54 plots in a systematic design without replication. These plots were arranged as nine blocks each containing six plots, with the resistant and susceptible genotypes sown in three plots of each block (Fig. 1). In addition, two buffer plots separated each experimental plot and a 2 m pathway separated each block of plots. Each year the trial was positioned so that four blocks of plots (24 plots) were on the positive soil and four blocks on the negative soil, with the middle block (six plots) placed on the border of these soils zones. The centre-line of each block was positioned at (±) 4, 8, 12 or

16 m from the border (designated as 0 m) within each positive and negative soil zone (Fig. 1). Each plot was 2.0 m long and consisted of a 'hedge-row' i.e. two adjacent rows spaced 25 cm apart. There was a 50 cm space between plots. Each plot was sown by hand to 4 cm sowing depth, with 18 seed sown in two rows of nine seeds spaced 25 cm apart. After sowing, the furrows were lightly scarified to ensure good seed-bed coverage. CLS was allowed to establish naturally and no fungicides were applied. Weather data, including daily rainfall and temperature, were recorded on site via an automated weather station and instrument readings by staff at the research station.

Incidence of CLS on faba beans sown into two soils with different crop rotations. In 2005, the trial was sown on 12 May and the incidence of disease on each plant was recorded weekly from 42 days after sowing (DAS) (30 June) to 112 DAS (1 September). In 2006, the trial was sown on 25 May and incidence data recorded from 56 DAS (19 July) to 119 DAS (21 September).

In each field trial described above, the disease incidence data (presence or absence) for individual plants were recorded on a spatial map in Windows® Excel. An individual cell in Excel represented an individual plant and these were subjected to formatting to characterise cell values with a colour to identify three time periods when disease was first observed on plants; 49-63 DAS, 70-84 DAS and 91-119 DAS. Maps were then constructed to visualise the spatial pattern of disease development on plants of both genotypes sown within the positive and negative soil zones for each of the three time periods in each year.

Severity of CLS on faba bean sown in two soils of different crop rotations. Commencing at the onset of disease, six plants in the centre of each experimental plot described above were assessed for; the number of nodes (leaves) on each plant on the main stem, the number of nodes with CLS lesions, the estimated Leaf Area Diseased (%LAD) on each node, the number of nodes from which leaves had abscised. In 2005, these data were recorded for all 54 plots between 49 and 84 DAS. Between 91 and 105 DAS one plot of each

genotype within each block was omitted to save time, resulting in 36 plots assessed, and in the final assessment at 112 DAS, all plots in blocks on the -4 m, 0 m, +8 m soil positions were excluded, resulting in 24 plots assessed. In addition, disease was recorded using time-lapse digital photography at weekly intervals between 63 and 98 DAS on one leaf-pair selected arbitrarily within plots sown ±12 m in the positive and negative soil zones. In 2006 the severity of CLS was assessed as described above in all 54 plots between 56 and 98 DAS, on 36 plots between 105 and 112 DAS, and in 24 plots in the final assessment at 119 DAS, omitting plots located on boundary (0 m) and in blocks located at ± 8 and ± 16 m positions, to balance assessments in each soil zone. Due to drought in 2006, the trial was irrigated once at 98 DAS using over-head sprinklers, providing water equivalent to a *ca* 25mm rain event.

Temporal disease severity curves were developed for both genotypes, and spatial disease severity curves across the two soil zones were developed for disease scores at 77 and 98 DAS in both genotypes. The loss of photosynthetic leaf area (%LPLA) was used to assess overall CLS severity. This was calculated as the sum of total %LAD and defoliation (equivalent to 100% LAD) and expressed as the % of total plant foliage affected by disease. No disease was recorded in the first 42 DAS in the 2005 trial, so only dates after this time are included in the analyses. Disease data (%LPLA) were square root transformed where required to normalize the data. The mean %LPLA for each genotype and soil zone position were plotted against DAS and data were fitted to exponential or logisitic standard curves using Genstat® version 11. The best fit model was determined by significance at $P \leq 0.05$, and the percentage of variance explained. The fitted values and residual mean square values (RMS) of %LPLA per genotype in each block were obtained for two time periods, 77 and 98 DAS. These time periods allowed examination of the largest data set. The mean standard error (SEM) values were calculated from the RMS values, which also accounted for data points at either side of the selected time periods (ie. 70 and 84 DAS for fitted value at 77 DAS). The estimated values at 77 and 98 DAS for each genotype and position between and within each

soil zone were compared using a two-sample *t*-test at P=0.05. Plots sown in the column positioned at +16 m were omitted from the main analyses as these were on the outer perimeter of soil that had been planted with faba bean every 3 years. Cumulative rainfall data recorded at each trial site between 49 DAS and 112 DAS were regressed against mean %LPLA on the susceptible genotype sown on the positive soil zone (+12 m position) and significance of the deviation from zero was determined at P<0.05.

Survival of *C. zonata* in soil. An experiment was conducted over 30 months from December 2005 to June 2008 at the Charlick Research Station (Latitude: 35.26°S, Longitude: 138.89°E), located 5 km south of Strathalbyn, South Australia. The region has a Mediterranean climate with mean annual rainfall 490 mm, 80% of which falls between April and October, elevation 70 m, and red-brown loam soil. The role of infested faba bean residue in survival of *C. zonata* was examined. Fungicides for control of CLS had been evaluated at this site and faba beans harvested in December 2005, resulting in deposition of *C. zonata*-infested crop residue on the soil. On 20 December, 2005, immediately following harvest, the residue trial was established over three untreated control plots of the fungicide trial, each estimated to have *ca* 600 g of infested residue on the soil surface. Each of the untreated plots was divided lengthwise to create three residue treatment plots (each 1.6 x 2 m). Hence the residue trial was arranged as randomized complete block design (RCBD) with three replicate treatment blocks, each with three treatment plots. Three treatments were established; faba bean residue retained on, or removed from, the soil surface, or plots grazed by sheep. The first two treatments were enclosed by 1-m high steel-mesh enclosure to exclude grazing animals. Weed infestation was controlled by application of glyphosate (10 ml/liter) when necessary. Thirty-five soil cores (2.5 cm diameter to 10 cm depth) were extracted from each treatment plot at the beginning of the trial (0 months) and every 3 months for 27 months (10 sampling times). The soil cores were oven-dried for 4 days at 40^{o}C, and placed in a cold store (4^{o}C). In June 2008, a 200 g sub-sample was extracted from stored soil cores for each

replicate x treatment x sampling time. Soil samples were sent to the commercial service provided by the RDTS (SARDI) (Ophel-Keller et al., 2008) for quantification of the DNA of *C. zonata* in the soil (pg DNA/g soil). This commercial service has been used in other studies (Herdina et al., 2004; Sosnowski et al., 2006; Wakelin et al., 2009; Bithell et al., 2009). Specific primers and probe for the real-time PCR were designed based on the highly conserved internal transcribed spacer (ITS) region of the ribosomal genome. The sequences (5' > 3') of the primers were CCGGTCTTCTGACCGGCTC (forward) and GGACCTCGCCCGAGTCT (reverse) and the probe was AGGCCATCATCGTCT, Taqman® MGB (Minor Groove Binder) with 6-carboxyfluorescein (FAM) at the 5' end and non-fluorescent quencher at the 3' end (Applied Biosystems, CA, USA). The specificity was evaluated using a range of isolates comprising both *C. zonata* and other fungi. The PCR conditions described by Riley et al. (2009) were used, except that the final concentration of primers was 1 mM and the annealing temperature was 55ºC for 60 s followed by extension at 72ºC for 30 s.

The relationship between soilborne inoculum and disease severity was tested for the soils sampled at 0, 9, 18 and 27 months from the 'retained' and 'removed' residue plots. The experiment was conducted in a pot-bioassay as a completely randomised design with three replicates. Soil with no history of cropping, collected from a site at the Charlick Research Station, was included as a control. Twenty-seven pots (15 cm diameter) filled with pasteurised University of California potting mix were sown with four seeds of cv. Farah at 3 cm depth, then covered with a 1 cm layer of soil from the appropriate treatment. Immediately after sowing, pots were watered to capacity then maintained outside (Waite Campus) under irrigation from 9 July to 3 September. The pots were placed 40 cm apart to minimise cross-contamination. Seedlings were assessed for disease 8 WAS, at the five node growth stage, as average %LAD on each leaf pair. Disease data were correlated with the quantity of DNA of *C. zonata* detected in corresponding soil samples.

After completion of soil sampling, 30 months after the harvest of the fungicide trial in 2005, the infectivity of inoculum in each plot was assessed *in situ*. The enclosures were removed and seven furrows, 25 cm apart along the length of each plot, were prepared by hand to minimise transfer of soil between plots. On 25 June 2008, each plot was sown with cv. Farah (20 seeds/m^2) at 4 cm depth. When seedlings reached five nodes, at 63 DAS (26 August), the average %LAD and defoliation (%) was assessed on the lower 50% of the canopy of plants within each plot, excluding those on the perimeter to avoid interplot interference.

DNA and disease data from the pot assay were transformed (square-root) where required for normality of data and subjected to ANOVA at common sampling times (0, 9, 18 and 27 months) using Genstat® version 11. Significant differences were separated at $P \leq 0.05$. Disease data from the *in situ* field assay were analysed using ANOVA and correlations between quantity of DNA per g of soil and mean %LAD per plant from the pot assay were assessed using GraphPad Prism® version 5.

RESULTS

Incidence of CLS on faba beans sown into two soils with different crop rotations. In 2005, CLS was first observed on the susceptible genotype (cv. Farah) sown in the positive soil zone at 49 DAS. The lesions were on the lowest two leaves (three to four node growth stage) of five seedlings randomly distributed through the plots, representing 3% of plants. In 2006, 72% of cv. Farah plants sown in the positive soil zone were diseased at 56 DAS, and assessments prior to this time were not possible. In both years, disease incidence increased rapidly, resulting in lesions on 90 to 100% of susceptible plants sown in the positive soil zone at 63 DAS (Fig. 1). Sporulation on primary leaf lesions was not observed until 1 to 2 weeks after symptoms were first observed, by which time lesions exceeded 8 mm in diameter.

CLS lesions on seedlings sown in the negative soil zone were first observed one week later than in the positive soil zone in both years. In 2005, this occurred at 56 DAS, with CLS appearing as small lesions on 18% plants of cv. Farah, of which 77% were within 8 m of the border with the positive soil zone. In 2006, CLS lesions were first observed in the negative soil zone at 63 DAS on 56% plants of cv. Farah, of which 63% were within 8 m of the border with the positive soil zone. Disease incidence on seedlings in the negative soil zone between 56 and 63 DAS in 2005, and at 63 DAS in 2006, decreased with distance from the positive soil zone (Fig. 1). Disease incidence on plants of cv. Farah located -4, -8, -12 and -16 m from the border with the positive soil zone at 63 DAS was 65, 35, 13 and 6%, respectively, in 2005 and 75, 65, 51 and 30%, respectively, in 2006. A temporal disease gradient was also observed in 2005 for the timing of maximum disease incidence (80-100%) on cv. Farah sown in the negative soil zone. These maxima occurred at 70, 77 and 84 DAS in plots positioned at -8, -12 and -16 m from the border with the positive soil, respectively.

The spatial patterns of disease incidence were similar on the resistant genotype (breeding line 1322/2), although incidence was substantially less. Disease was first observed in the positive soil zone between 49 and 63 DAS, on fewer than 5% of seedlings. Between 70 and 84 DAS, disease incidence increased to 62% (2005 trial) and 47% (2006 trial) in the positive soil, and to 18% (2005 trial) and 6% (2006 trial) on plants in the negative soil zone (Fig. 1). At 98 DAS, 93% (2005) and 89% (2006) of breeding line 1322/2 within the positive soil exhibited small lesions typical of CLS. A disease gradient for incidence from the source of inoculum was evident for breeding line 1322/2, though many plants remained symptomless, leading to a rapid decline in disease incidence in plots furthest from the positive soil zone (Fig. 1). At 98 DAS, disease incidence in plots sown -4 to -16 m from the border fell from 72% to 17% in 2005 and 32% to 6% in 2006, but was 100% in cv. Farah in plots sown in the same positions.

Severity of CLS on faba bean sown in two soils of different crop rotations. Disease (mean %LPLA ± SEM) was most severe (72% ± 6.0 in 2005, 53% ± 2.7 in 2006) on the lower 50% of the canopy on cv. Farah sown in the positive soil at 98 DAS, particularly on the three nodes (85% ± 4.3) closest to the soil surface, whereas disease was slight (2% ± 0.7 in 2005, 0.02% ± 0.02 in 2006) in the upper 50% of the canopy. Lesions expanded and coalesced from 49 to 98 DAS, causing significant necrosis (Fig. 2). In the most affected plots, the maximum mean %LAD was recorded approximately 98 DAS (i.e. 35% LAD in +4 m plots) (Fig. 2). Defoliation of the lowest infected leaves in the canopy due to CLS commenced at 77 DAS (Fig. 2) and this caused a decline in mean %LAD after 98 DAS. Therefore, data for LAD and defoliation were combined to calculate the total loss of photosynthetic leaf area (%LPLA) to quantify the severity of CLS. In both years, disease development on cv. Farah positioned centrally (12 m) within the positive soil zone showed a rapid increase in severity from 77 to 91 DAS. Disease did not increase substantially during this period on cv. Farah planted in the negative soil nor on the resistant breeding line 1322/2 in either soil zone (Fig. 3). In the positive soil zone, cv. Farah showed >10 %LPLA by 77 DAS, which progressed to >50 %LPLA by 98 DAS, resulting from both lesion expansion and defoliation during that period (Fig. 3). This effect was captured using time-lapse photography of CLS development on the susceptible genotype in 2005 (Fig. 4).

CLS on cv. Farah was more severe in 2005 (max. mean LPLA = 50%) than in 2006 (max. mean LPLA = 35%) in the positive soil. Rainfall was 29% above and 22% below the long term average for the entire year of 2005 and 2006, respectively (BOM, 2010). In 2005, 13 rain events that exceeded 5 mm were recorded between July and October, whereas in 2006, there were six such rain events (Fig 3.). There was a significant regression in 2005 (R^2=0.78; $P \leq 0.001$) and 2006 (R^2=0.74; $P \leq 0.001$) between disease severity (mean %LPLA) on the susceptible genotype in the positive soil zone (positioned at +12 m) and cumulative rainfall recorded each season between 49 DAS and 112 DAS. In both trials the increase in %LPLA

over time on the susceptible cv. Farah in the positive soil zone fitted a logistic curve (P<0.001). In each trial, disease was most severe in all plots positioned at +4, +8 and +12 m in the positive soil zone. In contrast, CLS (%LPLA) was significantly less severe on cv. Farah grown in the negative soil zone, and severity over time fitted an exponential curve (P<0.001). Disease was on the resistant genotype (1322/2) was slight in both soil zones (Fig. 3).

At the first selected assessment time (77 DAS), the %LPLA was attributed solely to %LAD, as this was prior to the onset of defoliation (Fig. 2). This was also the earliest time CLS was observed on either genotype sown in either soil zone. The second selected assessment time (98 DAS) represented the upper asymptote of disease severity on the susceptible genotype (cv. Farah) sown in the positive soil zone (Fig. 3).

The fitted values for mean %LPLA at 77 and 98 DAS calculated in non-linear regression analyses for the two genotypes in the nine blocks of plots are presented in Fig. 5A (2005) and Fig. 5B (2006). The paired two-sample t-test analyses of mean %LPLA across the soil zones showed that disease was significantly more severe on the susceptible genotype (cv. Farah) (P<0.001) in the positive soil zone than in the negative soil zone in both years (+ve versus –ve: Table 1) for both 77 DAS (2005 trial t = 11.33; 2006 trial t = 25.65, P<0.001) and 98 DAS (2005 trial t = 32.75; 2006 trial t = 41.49, P<0.001). In addition, disease severity was significantly different (P<0.001) for both times (77 and 98 DAS) when matched planting positions (e.g. –8 versus +8 m) in the two soil zones were compared (Table 1). In both trials, there was a substantial drop in disease severity in the negative soil within 4 m of the boundary with the positive soil zone (Fig. 5). In both years, CLS (%LPLA) was significantly (P<0.001) more severe (11%) at 77 DAS on plants at the edge of the positive soil zone (0 m) than on plants located 4 m away in the negative soil (4% in 2005, t = 5.34; 2% in 2006, t = 12.95), and on plants located 8 m away, where severity was similar to that at 4 m (Fig. 5; Table 1). Disease at the boundary (0 m) was also significantly more severe (P<0.001) at 98 DAS (33%

in 2005, 23% in 2006) than on plants located 4 m (12% in 2005, 7% in 2006) and 8 m (15% in 2005, 7% in 2006) away in the negative soil (Fig. 5; Table 1).

CLS on breeding line 1322/2 was considerably less severe than on cv. Farah in both 2005 and 2006 and did not exceed 5% LPLA, so few comparisons were possible between soil zones as data were less amenable to analysis. Nevertheless, significant (P<0.05) differences in disease were observed in both years at 98 DAS between plants sown in the negative soil zone (1% mean LPLA both years) and those in the positive soil zone (3% mean LPLA in 2005, 5% mean LPLA in 2006) (Fig 5). The difference in severity between the two soil zones was also significant (P<0.05) at 77 DAS in 2006 but not in 2005 (Table 1).

Survival of *C. zonata* inoculum in soil. The effect of stubble treatment on survival of *C. zonata* was examined over a 3-year period from 2006 to 2008, during which the average monthly rainfall and temperatures were below and above the long-term average, respectively, each year (BOM, 2010). At the first sampling time (3 months) there was little visible evidence of faba bean residue on the surface of 'grazed' plots. Conversely, in treatments where residue was 'retained', residue was visible on the soil surface after 27 months. While efforts were made to exclude residue from 'removed' treatment plots, small fragments were occasionally observed on these plots as a result of wind.

The quantity of DNA of *C. zonata* detected in the soil (pg/g soil) declined over time in all treatments, decreasing by 70-80% over the sampling period (Fig. 6). There was a significant effect (P<0.05) of residue treatment on the quantity of DNA of *C. zonata* detected in the soil samples. When residues were 'removed' from the soil surface immediately after harvest, the quantity of DNA of *C. zonata* detected in the soil samples was significantly less (P<0.05) at all sampling times than when residue was 'retained' (Fig. 6). At the early sampling times of 0 and 9 months, there was no significant difference (P>0.05) in the amount of DNA of *C. zonata* detected in soils where residue was 'grazed' or 'retained'. However,

soil samples collected from the 'grazed' plots at 18 and 27 months yielded significantly less (P<0.05) DNA of *C. zonata* than those where residue was 'retained'.

When the 'infectivity' of soil samples collected from the 'retained' or 'removed' plots at 0, 9, 18 and 27 months was tested on cv. Farah in a pot assay, CLS was first observed at 6 WAS. At 8 WAS CLS was significantly (P<0.05) more severe (>50% LAD) on seedlings exposed to 'stubble retained' soils than to 'stubble removed' soils (<15% LAD) (Fig. 7A). Plants exposed to the 'stubble retained' soils collected at the first sampling time were more severely diseased (P<0.05) than those exposed to such soil collected 9 months later after which disease severity changed little (P>0.05). Disease severity on plants exposed to 'stubble removed' soils (12.2% mean LAD) was statistically comparable (P>0.05) to that in the control soil (3.8% mean LAD) and changed little for the four times of sampling. Control plants exposed to soil not previously used for faba bean exhibited slight disease, possibly due to cross-contamination. There was also a significant positive regression (R^2=0.93; y = 0.09x + 12.31, P<0.05) between the infectivity of 'retained' soils in the pot-assay (% LAD on seedlings) and the quantity of DNA of *C. zonata* detected in those soils. However, this relationship was not demonstrated (R^2= 0.002; y = -0.89x + 1.12, P>0.05) for 'removed' soil treatments.

The *in situ* field assay, conducted 30 months after harvest of the 2005 field trial, resulted in significant (P<0.05) differences in disease among all three residue treatments (Fig. 7B). Disease at 9 WAS was more severe (P<0.05) on seedlings grown in 'retained' plots (48% LAD) than in 'grazed' plots (29% LAD) which, in turn, was more severe (P<0.05) than in plots from which residue had been 'removed' (18% LAD).

DISCUSSION

This study has shown a strong association between the incidence and severity of CLS and soil-borne inoculum. In both growing seasons, lesions typical of CLS appeared early (49

to 63 DAS) on the lowest leaves of seedlings of the susceptible faba bean genotype, with a sporadic spatial distribution in soil planted with faba bean every 3 years since 1997, designated the 'positive soil zone'. This spatial pattern, including the occurrence of lesions on leaves closest to the soil surface, has been reported for a wide range of soilborne pathogens (e.g. Campbell and Noe, 1985; Windels et al., 1998; Vereijssen et al., 2006). The incidence of primary infection foci in the susceptible genotype planted within the positive soil increased rapidly so that all plants exhibited lesions within 2 weeks. As the latent period of *C. zonata* on faba bean is 10 to 14 days (R. B. E. Kimber, *unpublished data*), this was considered to represent primary infection.

The temporal and spatial dynamics of cercospora leaf spot (CLS) on susceptible and resistant faba bean genotypes that resulted from soilborne inoculum in the field is described here for the first time. Non-linear regressions using a logistic curve best described disease progress over time on the susceptible genotype grown in soil where CLS inoculum had become established following regular cropping of faba bean, whereas an exponential model best described disease severity over time on the susceptible genotype at distance from the inoculum source and for the genotype exhibiting resistance to CLS in both soil zones. Both types of curves describe the development of plant disease epidemics (Zang et al., 2004; Madden et al., 2007) and provide useful information for disease management strategies, such as residue management and isolation of crops from residue infested in the previous years.

Disease on susceptible plants in soil that had no history of faba bean (negative soil zone) was observed a week later (56 DAS) than on the positive soil zone, at which time the incidence of diseased seedlings decreased with distance from the positive soil zone, constituting a disease gradient (Madden et al., 2007). The temporal and spatial patterns of disease incidence within the two soil zones indicated that primary inoculum that infected plants in the negative soil originated from the adjacent positive soil zone, either from splash-borne or wind-borne spores, rather than from foci within the negative soil. The alternative

explanation, that a lower level of inoculum was present in the negative soil zone, is rejected as, while this may have resulted in a similar spatial distribution, the time of disease onset would be expected to be similar in both soil zones (Vereijssen et al., 2006; Madden et al., 2007). That the incidence of CLS increased rapidly in the negative soil later in the season, after 77 DAS, reflects secondary infection (Vereijssen et al., 2007).

Defoliation in the lower 50% of the canopy, where CLS was most severe, in addition to lesion expansion (LAD), was an important component of disease severity on faba bean and, therefore, these measures were combined to describe the loss of photosynthetic leaf area (%LPLA) and quantify CLS severity. This association has been used to describe severity and yield loss caused by other cercospora diseases (Berger, 1977; Berger et al., 1997; Muro et al., 2001; Proulx and Naeve, 2009). Furthermore, at 98 DAS the defoliation observed on plants was attributed to CLS and occurred prior to other factors affecting defoliation, such as moisture stress or senescence, of the lower leaves. Disease severity (%LPLA) mid-season was greatest on plants that had exhibited primary infection early in the season (49 DAS), whereas disease was considerably less severe when primary infection occurred later (63 DAS). This suggests that the timing of primary infection is an indicator of potential loss of photosynthetic leaf area during the vegetative and reproductive stages of plant growth provided that conditions remain favourable for disease development. As such, a positive relationship between disease severity and accumulated rainfall between 49 and 112 DAS was demonstrated in both seasons, and drought conditions in 2006 resulted in reduced disease intensity compared with the wetter 2005 season.

C. zonata was dispersed from soilborne inoculum primarily over short distances. A distinct disease gradient was apparent for both the incidence and severity of CLS on susceptible and resistant plants sown 0 to 4 m from the positive soil zone; accordingly, the positive soil served as an ‘area source’ of inoculum, as defined by Madden et al. (2007). Maps of the temporal and spatial patterns of disease incidence and comparative analysis of

disease severity data, generated from non-linear regressions of the %LPLA, were used to identify significant differences at disease onset (49-63 DAS) and at 77-98 DAS on the susceptible faba bean planted in either soil zone. The gradients for CLS on faba bean were relatively steep in both years, shown by a reduction in disease incidence and severity on plants located 4 m from positive soil zone suggesting that dispersal of *C. zonata* from a source of primary inoculum was relatively limited. Furthermore, the principal agent for dispersal of *C. zonata* from soilborne inoculum is likely to be conidia, splashed by rain over short distances, to infect the lower leaves, and over larger distances by wind or wind-blown rain. Such mechanisms of dispersal and spatial patterns have been reported following field studies of other cercospora diseases (McKay and Pool, 1918; de Nazereno et al., 1993; Nutter et al., 1994; Vereijssen et al., 2006). In addition, steep within-field dispersal gradients have also been attributed to the relatively large conidia of *Cercospora* spp. (Ward et al., 1999). In our study, disease was observed 16 m from the border of the positive soil zone, indicating conidia may also be dispersed over substantial distances. In this context, a survey of commercial crops in southern Australia showed that incidence and severity of CLS were greater in crops adjacent to than remote from fields with infested residues (R. B. E. Kimber, *unpublished data*). Further investigation of the survival of *C. zonata* and dispersal over distances greater than 16 m is required to determine the influence of infested crop residues on subsequent infection of crops.

The severity of CLS on a susceptible faba bean was positively related to the amount of infested crop residue remaining on the soil surface. *C. zonata* survived in soil at least 30 months, at which time inoculum was capable of causing severe disease, findings which supported the results from the two field trials in which disease was observed on faba bean planted in soil that has been sown with faba bean every 3 years since 1997 (i.e. three to four crop cycles). Survival of other *Cercospora* pathogens on residue has been reported to range from 1-2 years (Latterell and Rossi, 1983) to several years (Nagel, 1938), but to decrease

dramatically when residues are buried (McKay and Pool, 1918; Khan et al., 2008). Therefore, while expansion of the area in southern Australia planted to faba bean over the last three decades and the susceptibility to CLS of current commercial cultivars (Kimber and Paull, 2011) may have contributed to the increased prevalence and severity of CLS in this region, the persistence of *C. zonata*-infested crop residues as a result of adoption of minimum tillage and incidence of drought is also likely to be important. Retention of crop residues on the soil surface has previously been associated with an increase in prevalence of disease and inoculum of other *Cercospora* pathogens (Payne and Waldron, 1983; Ringer and Grybauskas, 1995; Khan et al., 2008). Furthermore, the life cycle of many *Cercospora* species is poorly understood, though it has been suggested that if a sexual state does exist it would reside in the genus *Mycosphaerella* (Groenewald et al., 2006). Until the teleomorph of *C. zonata* is identified, critical aspects of the survival and dispersal mechanisms of primary inoculum will remain uncertain.

The analysis of CLS on faba bean reported here has demonstrated an association between soilborne inoculum from infested faba bean residue and the incidence and severity of CLS. This provides evidence of the role of close rotation (1-4 years) of faba bean crops in the increased incidence and severity of the disease in southern Australia (R. B. E. Kimber, *unpublished data*). The knowledge gained from this study can be used to profile CLS development in susceptible faba bean crops most at risk to the disease (i.e. in fields where inoculum is likely to be prevalent) and to optimise strategic foliar fungicide applications to control primary infection and reduce the loss of photosynthetic leaf area caused by the disease.

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Table 1: Comparative analyses of cercospora leaf spot (CLS) severity (mean loss of photosynthetic leaf area, %LPLA) on susceptible (cv. Farah) and resistant (1322/2) genotypes in two field trials conducted in 2005 and 2006 to examine the effect of soilborne inoculum. Each trial spanned a positive soil zone (+) sown to faba bean every 3 years, and an adjacent negative soil zone (-) with no history of faba bean crops. CLS severity on plants in each soil zone was estimated using fitted values at 77 and 98 days after sowing (DAS), calculated in non-linear regressions. Two sample paired *t*-tests were applied to the estimated values to determine significance differences in CLS severity between soil zones and between described distances (m) from the boundary (0 m) of the positive and negative soil zones.

Field Trial	Genotype	Soil Zone Positions*	(Sum of SE)^2	Difference in fitted %LPLA at 77 DAS	*t* statistic at 77 DAS	*P* value	Difference in fitted %LPLA at 98 DAS	*t* statistic at 98 DAS	*P* value
2005	Susceptible	+ve vs -ve	0.928	10.91	11.33	<0.001	31.55	32.75	<0.001
		+12m vs -12m	2.343	9.22	6.02	<0.001	39.17	25.59	<0.001
		+8m vs -8m	0.459	16.17	23.87	<0.001	27.83	41.09	<0.001
		+4m vs -4m	1.850	7.45	5.48	<0.001	33.17	24.39	<0.001
		0m vs -4m	1.522	6.59	5.34	<0.001	20.66	17.69	<0.001
		0m vs -8m	1.364	6.10	5.22	<0.001	17.51	14.19	<0.001
2005	Resistant	+ve vs -ve	0.078	0.33	1.20	ns	1.89	6.76	<0.001
		+8m vs -8m	0.031	0.97	5.52	<0.001	2.76	15.75	<0.001
		+4m vs -4m	0.239	-0.25	-0.50	ns	1.03	2.10	<0.05
2006	Susceptible	+ve vs -ve	0.317	14.44	25.65	<0.001	23.35	41.49	<0.001
		+12m vs -12m	0.246	15.43	31.14	<0.001	24.95	50.35	<0.001
		+8m vs -8m	0.524	16.54	22.85	<0.001	25.10	34.67	<0.001
		+4m vs -4m	0.435	15.32	23.22	<0.001	21.51	32.60	<0.001
		0m vs -4m	0.473	8.91	12.95	<0.001	16.09	23.40	<0.001
		0m vs -8m	0.558	9.80	13.11	<0.001	16.84	22.54	<0.001
2006	Resistant	+ve vs -ve	0.114	0.82	2.43	<0.05	4.57	13.56	<0.001
		+8m vs -8m	0.044	0.73	3.48	<0.01	5.76	27.61	<0.001
		+4m vs -4m	0.374	0.66	1.08	ns	3.09	5.05	<0.001

* *t*-tests were conducted between the fitted values for the two soil zones listed. n=24; $t_{24}(0.05) = 2.064$, $t_{24}(0.01) = 2.797$, $t_{24}(0.001) = 3.745$, ns = not significant.

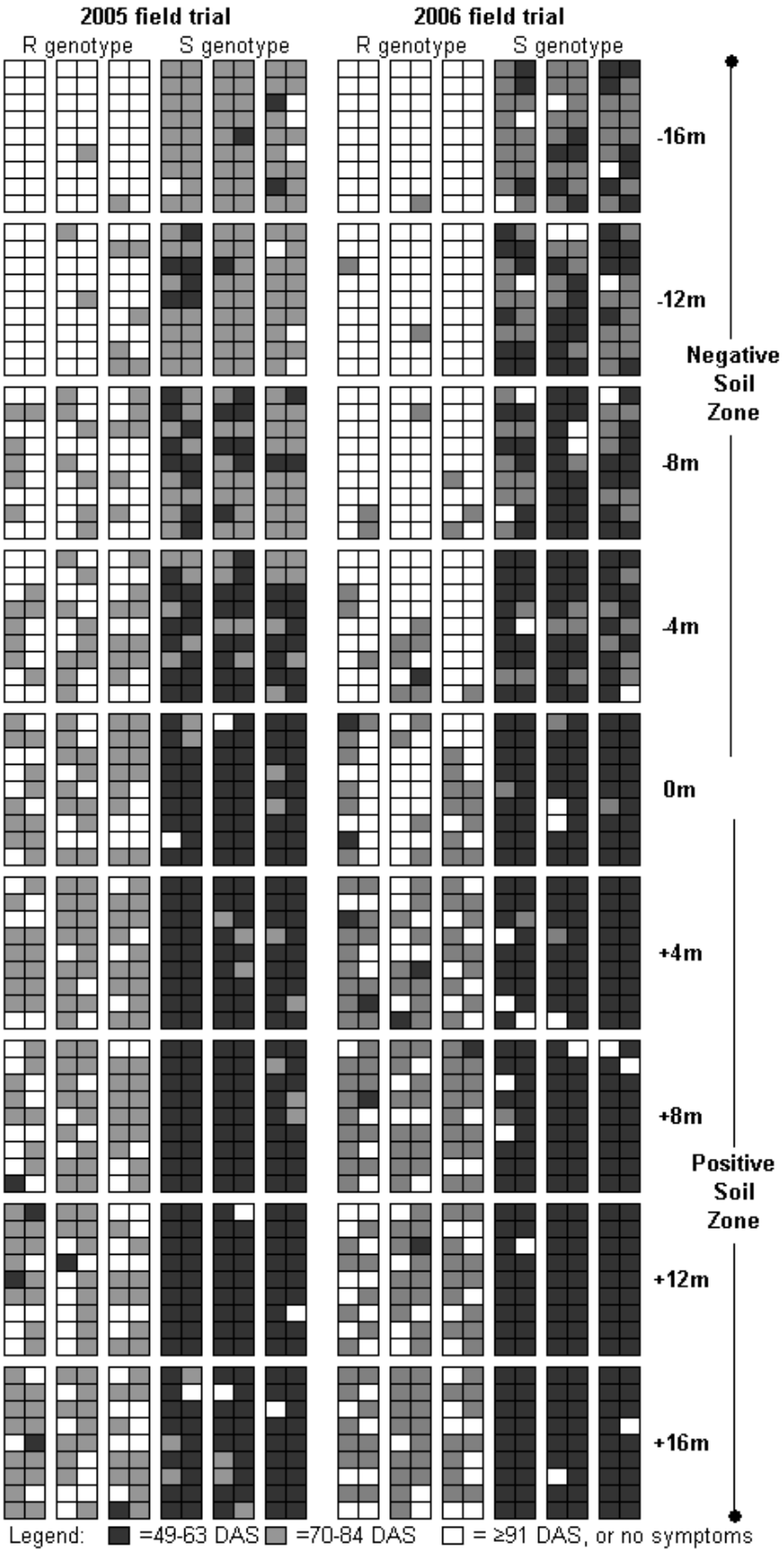


Figure 1: Temporal and spatial patterns of incidence of cercospora leaf spot (CLS, *Cercospora zonata*) in 54 plots (18 plants per 2.0 m hedge-row plot) of resistant (R) (1322/2) and susceptible (S) (cv. Farah) genotypes of faba bean (*Vicia faba*) in two field trials (de-

randomised) conducted at the Turretfield Research Centre, South Australia, in 2005 and 2006. Each pixel represents one plant, grown in soil with a 3-year rotation of faba bean (positive soil zone) or soil with no history of faba bean (negative soil zone). Plots were aligned on the boundary (0 m) of the positive (+) and negative (-) soil zones and at each of four distances (±4, 8, 12 and 16 m) from the boundary. Each pixel is shaded to indicate the time (days after sowing, DAS) when CLS lesions (incidence) were first observed (as indicated in the legend).

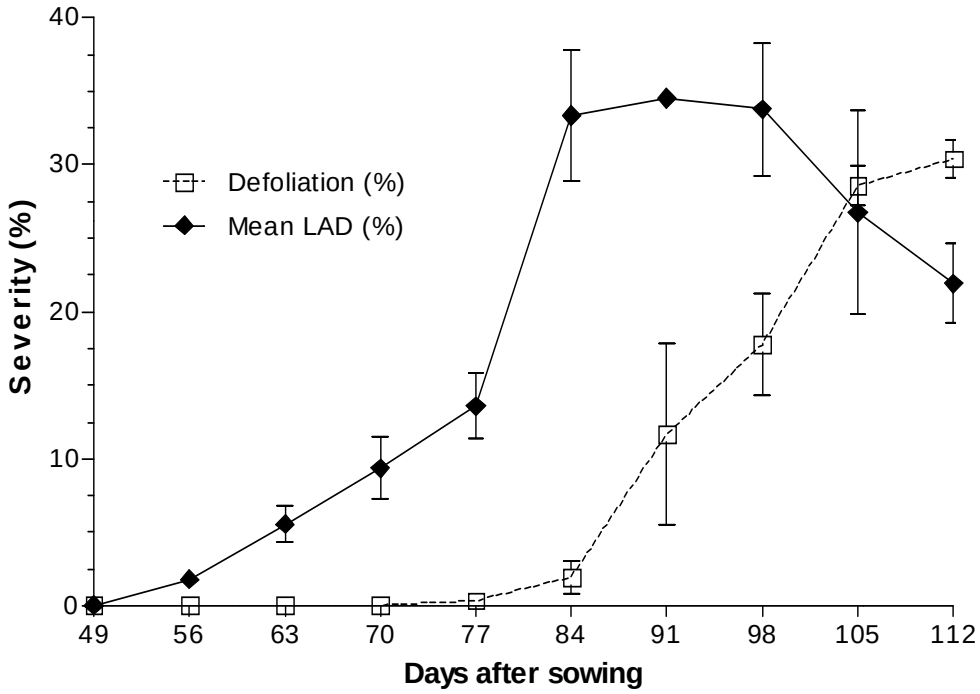
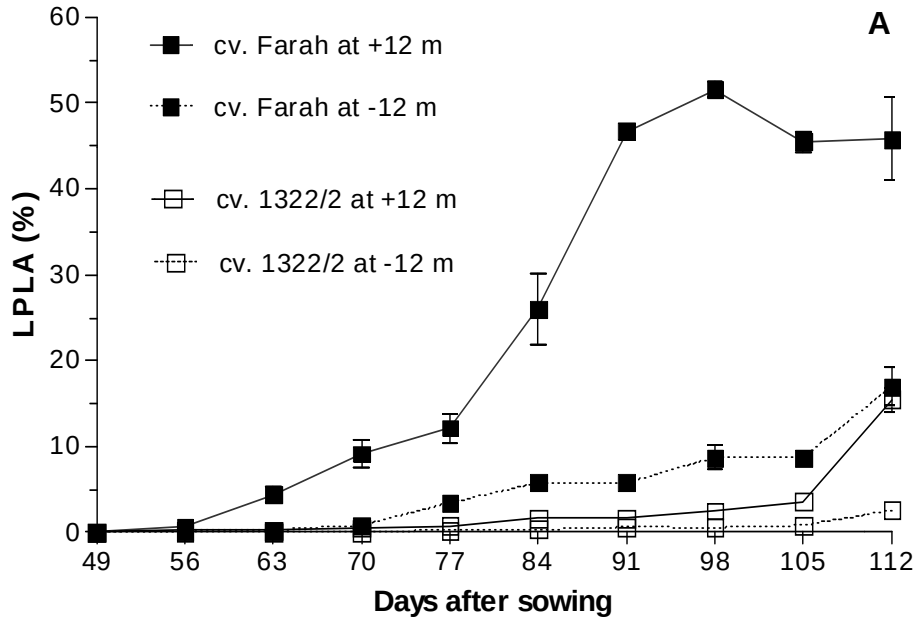
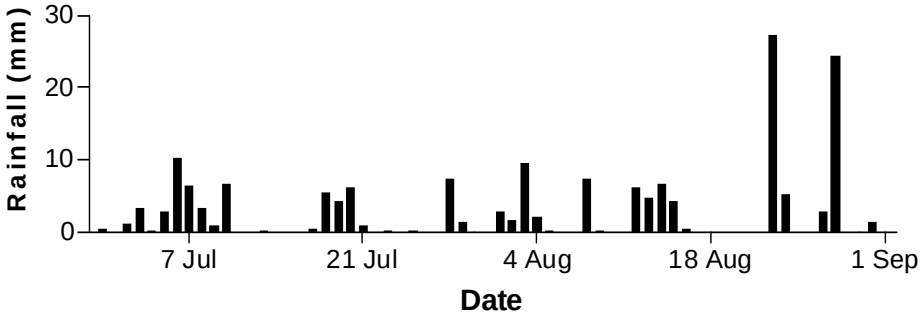


Figure 2: The severity of cercospora leaf spot (*Cercospora zonata*) on faba bean (*Vicia faba*) over time (days after sowing) measured separately as the Leaf Area Diseased (%LAD) and mean defoliation (% leaves abscised) on plants of cv. Farah (susceptible) grown in soil where faba beans were grown every 3 years (positive soil). Plants were in plots 4 m inside the positive soil zone (+4 m). Bars represent standard error of means (SEM).


A
cv. Farah at +12 m
cv. Farah at -12 m
cv. 1322/2 at +12 m
cv. 1322/2 at -12 m
LPLA (%)
60
50
40
30
20
10
0
49
56
63
70
77
84
91
98
105
112
Days after sowing


Rainfall (mm)
30
20
10
0
7 Jul
21 Jul
4 Aug
18 Aug
1 Sep
Date

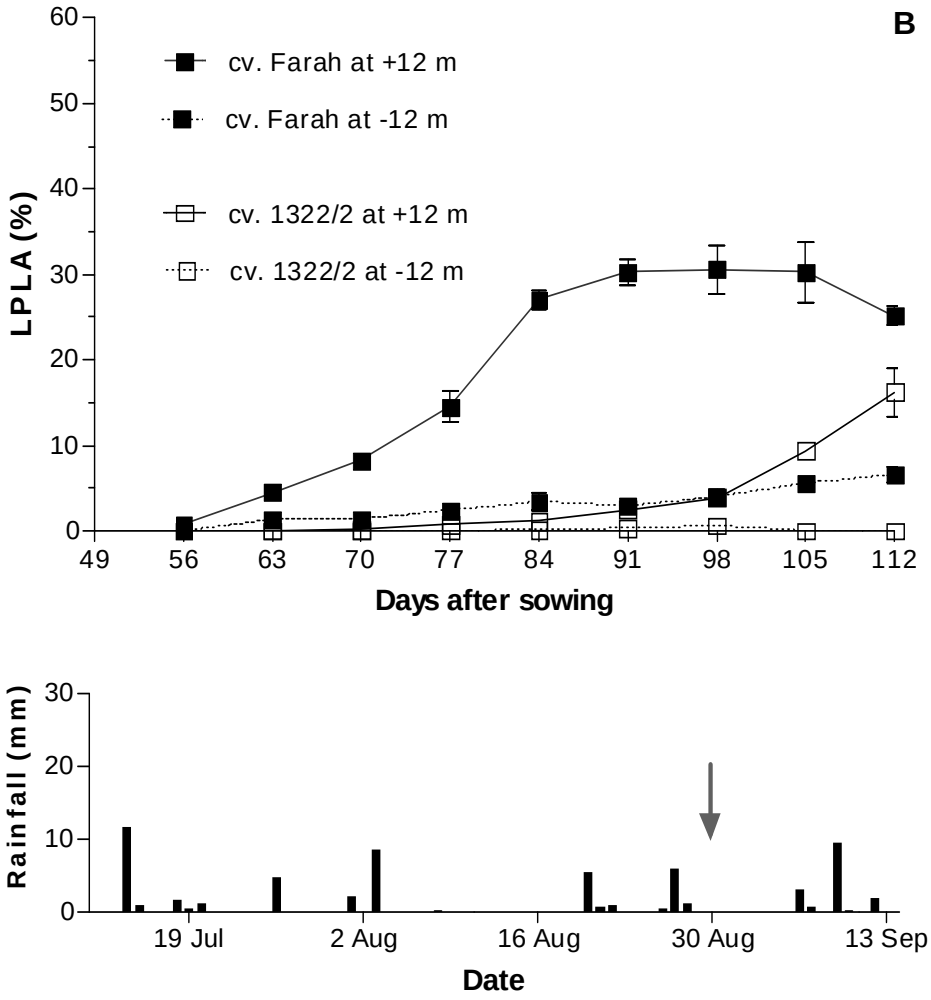


Figure 3: Severity of cercospora leaf spot on two faba bean (*Vicia faba*) genotypes, cv. 1322/2 (resistant) and cv. Farah (susceptible), grown in positive (+) soil zone, where faba bean was grown every 3 years, and in negative soil (-), without a history of faba bean cropping. Disease severity, measured as the loss of photosynthetic leaf area (% LPLA), was recorded each week from 49 to 112 days after sowing (DAS) in two field trials conducted in **A**, 2005 and **B**, 2006. Rain events were recorded during the same time period. The plots for each genotype were positioned near the centre of each soil zone (12 m from the centre of the trial). The gray arrow indicates an irrigation event in 2006 of an equivalent 25 mm rain event. Bars represent standard error of means.

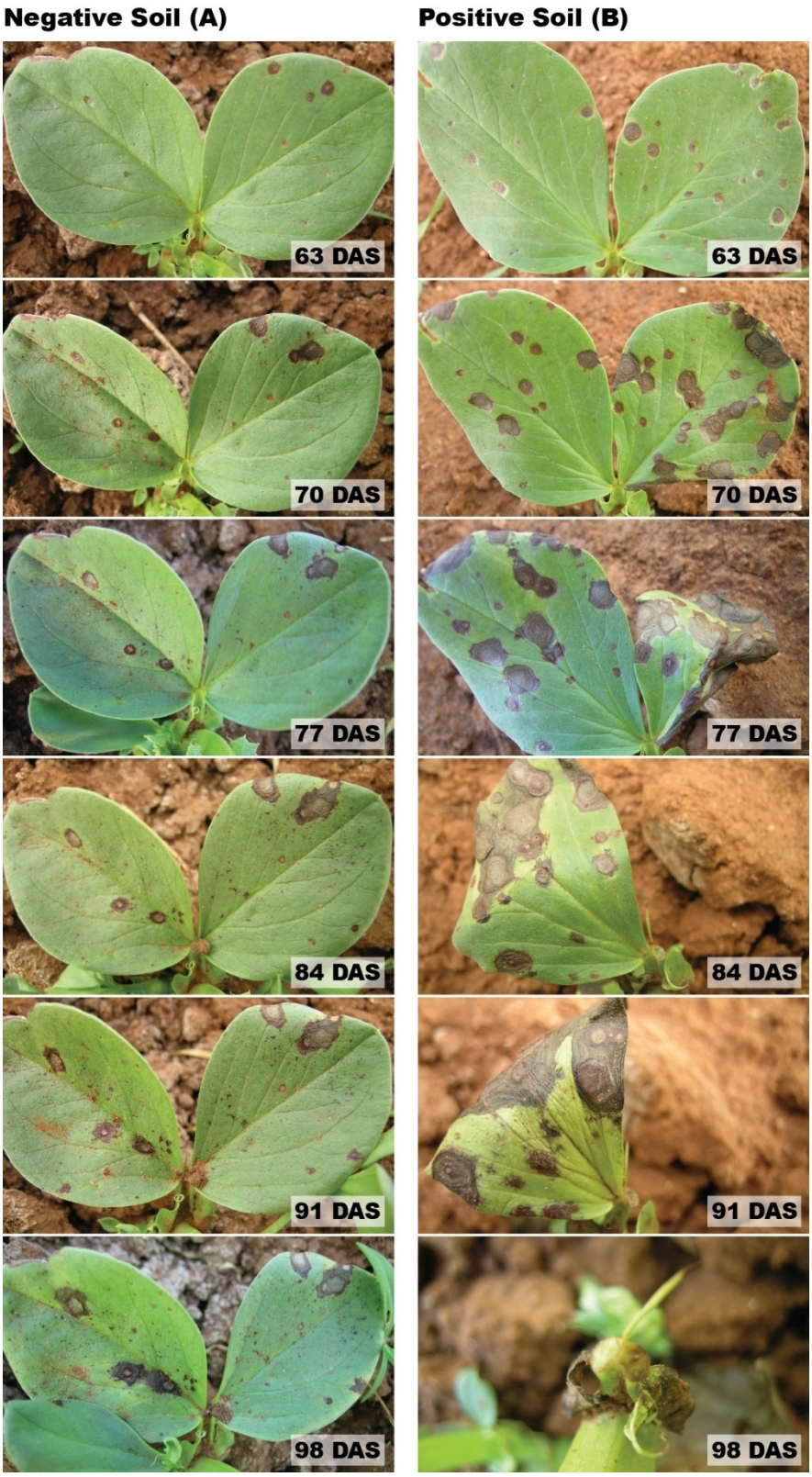


Figure 4: Cercospora leaf spot (*Cercospora zonata*) recorded each week (days after sowing, DAS) using time-lapse photography of the same leaf-pair in the lower-canopy of faba bean (*Vicia faba*) cv. Farah (susceptible) grown in; **A,** negative soil, with no history of faba beans, and **B**, positive soil, sown every 3 years with faba bean.

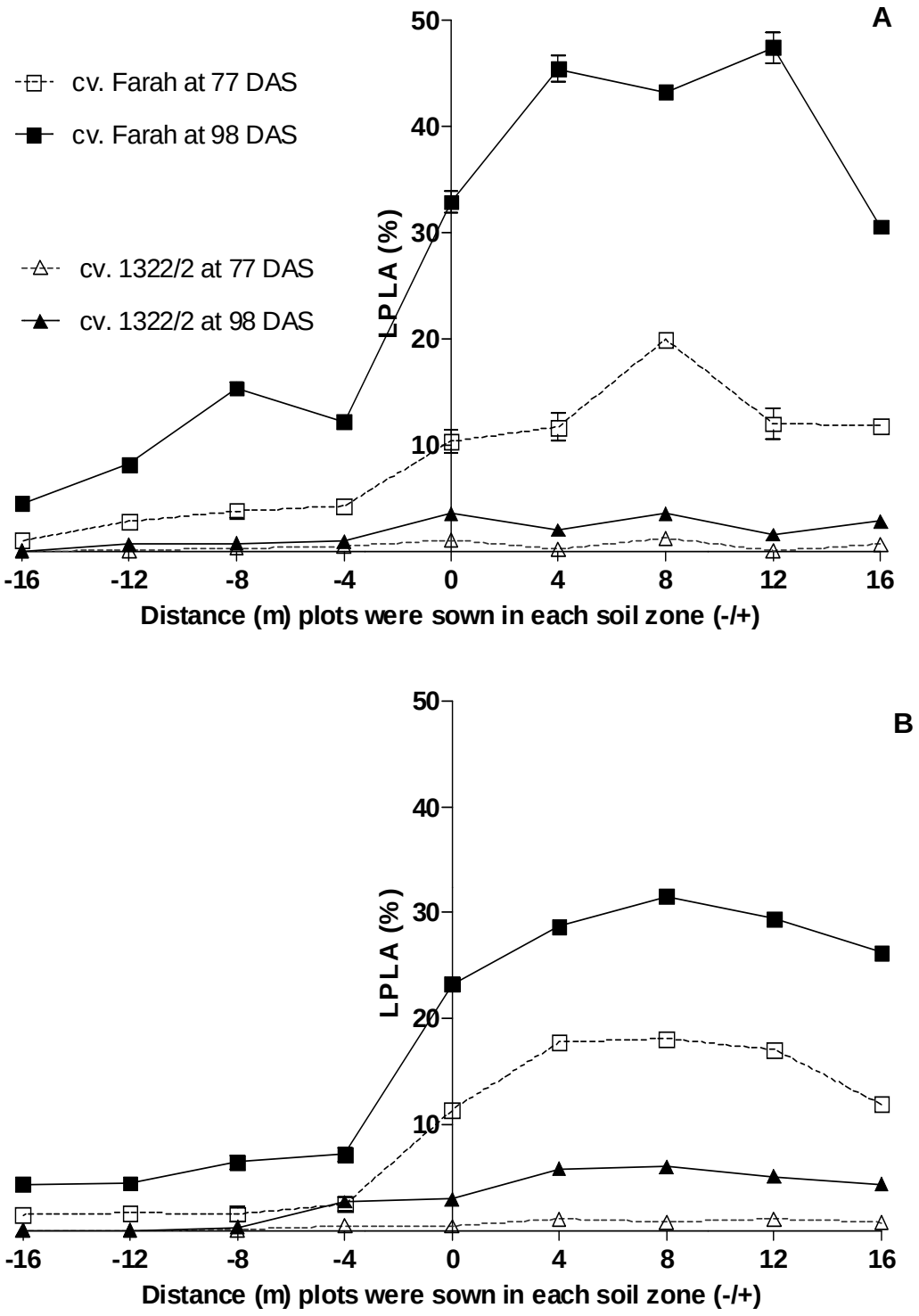


Figure 5: Cercospora leaf spot severity (mean percentage loss of photosynthetic leaf area LPLA) in **A**, 2005 and **B**, 2006, described using fitted values calculated from non-linear regressions at 77 and 98 days after sowing (DAS) for breeding line 1322/2 (resistant) and cv. Farah (susceptible) to the disease, planted at four distances (4 to 16 m) in a positive (+) soil zone, where faba bean was grown every 3 years, and a negative (-) soil zone, with no history of faba bean cropping, and on the boundary (0 m) that separated the two zones. Bars indicate standard error of the mean.

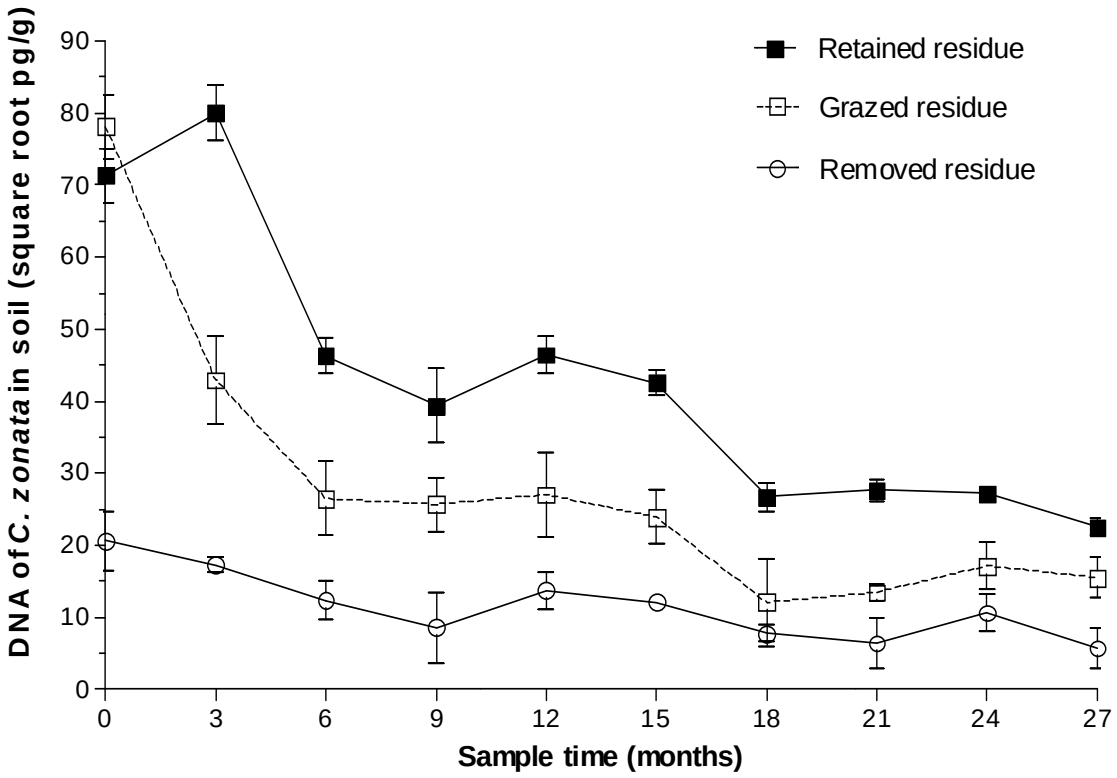


Figure 6: The effect of retaining, removing or grazing faba bean residue affected by cercospora leaf spot, on the quantity of DNA (pg/g soil) of *Cercospora zonata* detected in ten soil samples collected every 3 months from a field trial at Charlick Research Station, South Australia from 2005 to 2008. Data were transformed (square root). Bars represent mean standard error. $LSD_{0.05}$ (sample time); 0 months = 18.8, 9 months = 19.5, 18 months = 11.2 and 27 months = 4.8 √pg/g soil.

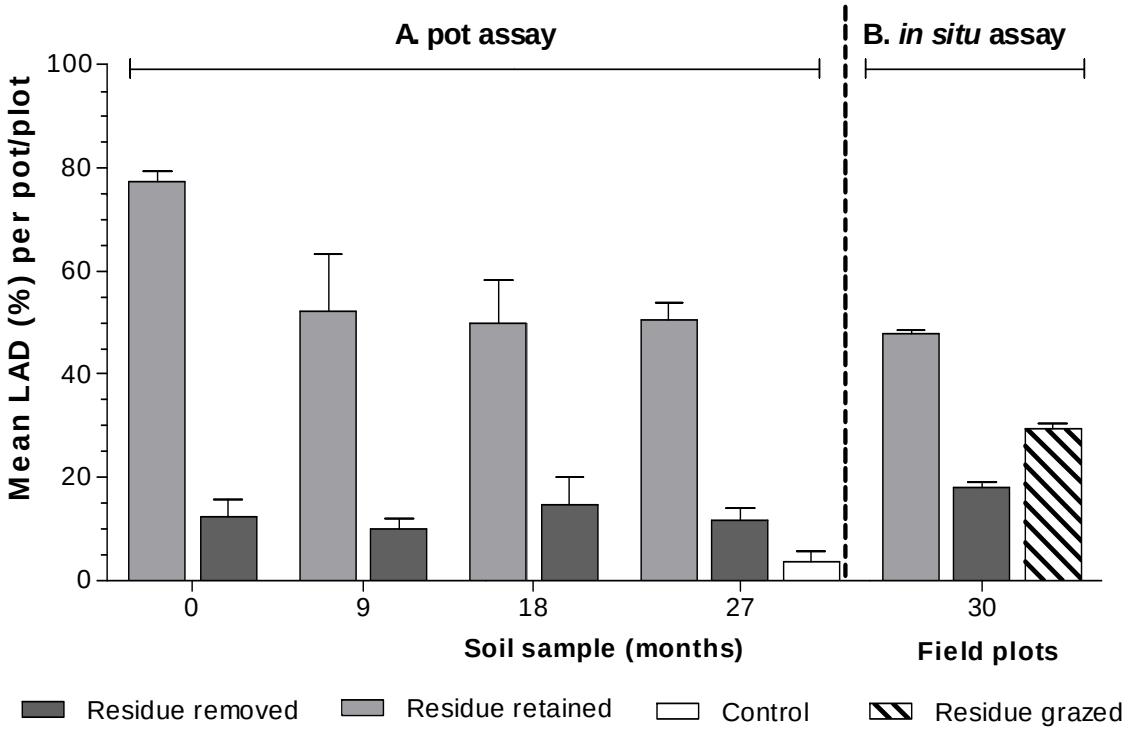


Figure 7: Mean Leaf Area Diseased (%LAD), caused by cercospora leaf spot (CLS), on faba bean seedlings (cv. Farah) grown in: **A**, a pot assay using soil collected 0, 9, 18 and 27 months from field plots after CLS-affected residue had been 'retained' or 'removed' from the soil surface; $LSD_{0.05}$ = 12.3, and **B**, an *in situ* assay where faba beans were sown in field plots, where residues had been retained, removed or grazed by sheep, 30 months after harvest; $LSD_{0.05}$ = 2.9. Control = soil collected near trial site but with no history of faba bean cropping. Bars indicate mean standard error.

Chapter 8.

Identification and genetics of resistance to cercospora leaf spot (*Cercospora zonata*) in faba bean (*Vicia faba*)

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Euphytica, v.177 (3), pp. 419 -429

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It is also available online to authorised users at:

http://dx.doi.org/10.1007/s10681-010-0288-7

Chapter 9.

General discussion

GENERAL DISCUSSION

This study has provided new information on the interactions between *C. zonata* and faba bean, and the factors that influence development of cercospora leaf spot (CLS). The epidemiology of the fungus and factors that affect the incidence and severity of the disease were examined, and the prevalence and significance of the disease in southern Australian faba bean crops established. The impact of CLS on faba bean yield was reported for the first time and management strategies to control the disease were developed for faba bean growers in southern Australia. Furthermore, a method to screen germplasm collections for resistance to *C. zonata* was developed, resistant genotypes were identified and the mode of inheritance of resistance was established.

In controlled environments, *C. zonata* caused slight disease on barrel medic, narbon bean, lentil and vetch, though pathogenicity was less severe than on faba bean. The biological significance of this finding is uncertain until the pathogen has been isolated from those plants in the field. However, the ability of *C. zonata* to 'passage' through other crop species may influence the survival and distribution of *C. zonata* in fields or growing districts where faba bean is not commonly cultivated; disease may then develop rapidly when faba bean is included in crop rotations (Woolhouse et al., 2001). In addition, multiple hosts may also influence fitness or aggressiveness of *C. zonata*, generating variation in the virulence of the pathogen (Regoes et al., 2000; Woolhouse et al., 2001; Zhan et al., 2002). Variation in aggressiveness was demonstrated amongst *C. zonata* isolates collected from various regions within southern Australia between 1999 and 2008. Though it is unclear what factors might contribute to variability in this pathogen, aggressive isolates were more common among those collected in 2005-2008 than in 1999-2004. This apparent increase in aggressiveness of *C. zonata* on faba bean after 2004 corresponds to the onset of increased prevalence of CLS in faba bean crops in southern Australia. However, further research is required to investigate

virulence patterns of *C. zonata*, including inoculation of a range of genotypes of faba bean and alternative hosts identified in this study, to elucidate the factors that might affect pathogenic variability. Extending the studies to molecular characterisation may establish if pathogenic variation corresponds to genetic diversity of *C. zonata* populations (Joshi et al., 2006).

Investigations of the effect of environmental conditions on the severity of CLS also provided a greater understanding of the dynamics of this disease in the field. Warmth (20-25^{o}C) in association with moisture was favourable to *C. zonata,* leading to rapid expansion of lesions on infected faba bean leaves and premature defoliation. Defoliation as a result of infection by *C. zonata* was greatest when the pathogen was inoculated at the mid- to late-vegetative stage of faba bean plant growth. The mid- to late-vegetative stage is acutely sensitive to stress, possibly the result of disruption to the hormone balance in mature leaves (Karamanos, 1978). The faba bean growing regions of southern Australia typically receive rainfall in association with maximum daily temperatures around 20^{o}C when crops are at the mid- to late-vegetative stages of development (BOM, 2010) and this may provide optimal conditions for CLS development. Overall, climate data recorded between 2002 and 2009 showed that most production regions in southern Australia experienced above average temperatures during the growing season compared to the long term average (BOM, 2010) and this may have also promoted development of CLS. However, *C. zonata* also infected faba bean at cooler temperatures in controlled environments (7-15^{o}C), which would allow the early onset of CLS in the field, but a long latent period was apparent at these temperatures, which would limit the number of infection cycles. This indicates that the number of primary infections may be more important than the number of secondary cycles in determining the loss of photosynthetic leaf area (LPLA) caused by premature defoliation and lesion expansion at warmer temperatures (Ringer & Grybauskas, 1995; Paul & Munkvold, 2005). Since LPLA was concluded to be the main factor that reduces yield of faba bean, practices that delay

disease onset and reduce the incidence of primary infections should minimise yield loss (Waggoner & Berger, 1987). This was demonstrated in field studies reported here using foliar fungicides applied strategically during the early developmental stages of CLS. Further studies to examine the timing of onset and tolerance of defoliation in faba bean may allow identification of disease thresholds that can be used to refine fungicide application strategies (Muro et al., 2001; Proulx & Naeve 2009).

Soil-borne inoculum is a major factor affecting CLS epidemics. In this study a strong association between the incidence and severity of CLS and soil-borne inoculum was established; a similar association has been reported for other cercospora diseases (McKay & Pool, 1918; Ward et al., 1999; Vereijssen et al., 2006). Therefore, if the level of initial inoculum is high and conditions are favourable for primary infection, disease may be severe even when few secondary cycles occur (Paul & Munkvold, 2005). Experiments conducted in the field showed that the quantity of CLS-infested debris remaining on the soil surface was a predominant factor in the availability of *C. zonata* inoculum to infect faba bean planted in subsequent seasons. The earliness and severity of primary infections were influenced by inoculum spread primarily over short distances during the early stages of CLS epidemics. Cultural practices that reduce the amount of CLS-infested residue on the soil surface, such as burial or grazing of residues, in addition to strategic fungicide applications that manage primary infections, are methods that may reduce the inoculum potential in high-risk fields and reduce the severity of CLS epidemics (Ringer & Grybauskas, 1995). However, a soil bioassay developed in this study showed that propagules of *C. zonata* captured in soil filtrates were too small (predominantly 15 to 105 μm) to represent infested organic matter, indicating the pathogen can survive in soil rather than exclusively on infested debris. This may explain why primary inoculum of CLS appeared to survive in fields with long periods between faba bean crops (>4 years), which is generally enough time to allow decomposition of infested residue (Dyke & Prew, 1983; Marcroft et al., 2003). In addition, a new procedure to detect

and quantify *C. zonata* in soil, based on a commercially available test to detect DNA of target soilborne pathogens (Ophel-Keller et al., 2008), was used in this study to demonstrate the longevity of CLS inoculum in soil and the 'infectivity' of this inoculum source was validated using the bioassay. Nevertheless, further validation of the sensitivity of this procedure, particularly to detect CLS inoculum in soils after longer periods of time, is required before this procedure can be considered to have commercial application.

Analyses of temporal and spatial patterns of disease spread from primary infections sites showed that spore dispersal exceeded 16 m from the area source of inoculum. In addition, results from the survey of commercial crops showed that disease incidence increased in crops when they were adjacent to fields containing infested residue. This implies that inoculum may also be disseminated over greater distances by wind, either as conidia, or as dry-blown particles of infested residue or soil-borne inoculum, as reported for other cercospora diseases (Carlson, 1967; de Nazareno et al., 1993; Nutter et al., 1994; Ward et al., 1999; Vereijssen et al, 2006). This could explain the incidence of disease in crops with no history of faba bean or residue in adjacent fields. Alternatively, on-farm activities and the movement of agricultural machinery, or the presence of other hosts of *C. zonata*, may also influence the timing and location of infections (Vereijssen et al., 2007; Suffert et al., 2011). It is likely, however, that the longevity of CLS inoculum, combined with the potential of inoculum to be dispersed between fields, has culminated in a build-up of soilborne inoculum in intensive faba bean production districts and increased the prevalence of the disease. This may explain why the south-east of South Australia, which has a long established history of CLS, exhibited more severe disease in crops than other growing districts in southern Australia, where CLS was more recently established. A similar finding was reported for CLS on sugar beet (*C. beticola*) in different growing districts in the Netherlands (Vereijssen et al., 2006). Therefore, CLS may increase in severity in susceptible faba bean crops grown in southern Australia if production intensifies or rotation time between faba bean crops is

reduced. However, the distribution of inoculum in soil of commercial fields was not examined in this study and quantitatively profiling local inoculum in fields in future disease surveys would offer valuable information on the distribution and spatial patterns of this disease.

The complete life cycle of *Cercospora* species is not well understood, but the few species for which a sexually reproducing stage (teleomorph) has been identified belong in the genus *Mycosphaerella* (Groenewald et al., 2006; Joshi et al., 2006). In the current study, two dispersal patterns of *C. zonata* were evident; inoculum dispersal over short distance that initiated primary infections, and inoculum dispersal over greater distances during the early stages of the epidemic. It is possible that this reflects dispersal patterns of conidia and ascospores. The teleomorph of fungal pathogens, such as those in the genus *Mycosphaerella*, can play an important role in generating air-borne spores capable of long distance dispersal and, in addition, sexual recombination may also be a significant factor in determining pathogen diversity and population structure and the adaptive potential of the pathogen (McDonald & Linde, 2002; Tivoli & Banniza, 2007; Suffert et al., 2011). However, until the sexual stage of *C. zonata* is identified, possibly on an alternative host(s), the biological mechanisms for the survival and dispersal of the pathogen will not be fully understood.

Genotypic resistance in *V. faba* also influenced the onset of epidemics as well as the progression of CLS. An important finding in this study was that all cultivars commercially available to the Australian faba bean industry were susceptible to CLS (Kimber & Paull, 2011). This undoubtedly has influenced the increased prevalence of the disease and contributed to a build up of disease inoculum within intensified cropping regions, irrespective of cultivar selection by faba bean growers. In contrast, the onset of CLS was delayed by 3 to 5 weeks in resistant genotypes, and both the rate of disease development and overall severity of the epidemic were reduced. The development of a rapid screening technique in controlled environments allowed the identification of several resistant genotypes in Australian

germplasm collections, and the establishment of a monogenic dominant mode of inheritance for resistance to *C. zonata* has provided the Australian faba bean breeding program with a relatively simple pathway to incorporate resistance into adapted genotypes (Baker et al., 1999; Kimber & Paull, 2011). These cultivars should be available to the southern Australian faba bean industry by 2016.

The mechanisms of resistance to CLS in faba bean were not examined, nor were the mechanism(s) by which *C. zonata* might trigger defoliation in faba bean. Many *Cercospora* species produce a non-specific phytotoxin called cercosporin, a photosensitising metabolite that plays a major role in pathogenesis (Fajola, 1978; Daub & Ehrenshaft, 2000). Increasing concentrations of cercosporin have been shown to correspond to increasing severity of CLS symptoms in the host and variation in toxin production among isolates of some species of *Cercospora* was linked to variation in aggressiveness on the host (Fajola, 1978; Daub & Ehrenshaft, 2000). The production of phytotoxic metabolites in populations of *C. zonata* should be examined to determine if they can be used to characterise the variation in aggressiveness (Tessmann et al., 2008) or the variability in symptom expression in faba bean genotypes reported in this study. In addition, such experiments could establish if cercosporin plays a role in the incidence of defoliation, since lesions were not always visible on infected petioles or at the point of abscission, or if cytotoxicity may be enhanced by the imposition of abiotic stress on the infected host, such as water stress due to drought, as has been experienced in southern Australia from 2001 to 2007 (Karamanos, 1978; BOM, 2007).

CLS was shown to cause yield loss in faba bean and the extent of damage was dependent on time of initiation, progress and severity of the epidemic. Foliar fungicides that suppressed the intensity of lesions on foliage and reduced defoliation were identified, and an economical application strategy for control of CLS in faba bean in the context of the yield loss potential from the disease was developed. This strategic approach was formulated using results from field trials that showed the onset of CLS on susceptible faba bean was 49 days

after sowing (DAS). Furthermore, *C. zonata* was shown to have a long latent period (14-20 days) at the temperatures experienced during the period in which these infections appeared (June to July, mean min-max, 9-16^{o}C). This led to the conclusion that infection of faba bean by *C. zonata* is likely to have occurred at approximately 35 DAS, and that a single application of prophylactic fungicide between 35 and 49 DAS can reduce the incidence of primary infection and delay the development of CLS in the crop. In contrast, temporal and spatial patterns of uncontrolled CLS demonstrated that when primary infections became established early on susceptible plants (49 DAS), disease severity increased rapidly during the vegetative stages of plant growth (77 to 98 DAS), presumably aided by the occurrence of several cycles of secondary infection during this time, and resulted in 30-50% LPLA. This pattern is characteristic of a polycyclic disease (Madden et al., 2007; Vereijssen et al., 2007). This information provided the basis for new fungicide recommendations to faba bean growers in southern Australia for the management of CLS (Hawthorne et al., 2011).

The results from the foliar fungicide efficacy trials provided new information that led to a permit approval for tebuconazole, of the conazole chemistry group, to be applied to faba bean to control CLS (APVMA, 2008). This provided another fungicide to be used in mixtures or alternations of active materials from different groups to be utilised by Australian faba bean growers for disease control, which would reduce the risk of fungicide insensitivity developing in pathogen populations due to the reliance on one fungicide chemistry group (Stoddard et al., 2010). However, only fungicide products that were registered in Australia for use on pulse crops at the time of this study were evaluated for efficacy against CLS on faba bean. Further research to examine the efficacy of alternative fungicide chemistries, including systemic fungicides that might be applied as seed dressings or in-furrow treatments, is recommended. Effective seed treatments can protect seedlings from primary infection by fungal pathogens during emergence and the early stages of plant development (Edgington et

al., 1980) and this may provide an alternative to an early application of foliar fungicide to control CLS on faba bean.

In conclusion, CLS was endemic in the major faba bean growing districts of southern Australia examined and was the most prevalent fungal disease of faba bean observed in 2007. In a short time, the disease has become widespread across the major faba bean production regions in Australia. This study has provided new information on CLS and provided the basis for management practices that can reduce the impact of the disease in commercial crops. Prior to the findings reported in this thesis, knowledge of factors affecting infection of faba bean by *C. zonata*, epidemiology of CLS, and the significance of the disease to faba bean production was poor or even lacking. As discussed in this thesis, several factors may have contributed to the emergence of the disease in southern Australia. First, all commercially available cultivars grown in southern Australia are susceptible to CLS, and incorporating resistance into future cultivars has now become a priority. Second, inoculum of *C. zonata* appears to survive in soil for several years and to increase rapidly in fields when faba bean rotations are reduced and when infested residues are retained on the soil surface. Third, the faba bean industry in southern Australia has become established over the last three decades and the area planted to the crop is now 115,000 ha, double that planted 15 years earlier (ABARES, 2011). This would have compounded the build-up of CLS inoculum, as more growers have included faba bean in regular rotations in their fields. Finally, incorrect diagnosis of the disease led to inappropriate use of fungicides early in the growing season, which allowed the disease to establish. New recommendations for cost effective strategic applications of effective fungicides have now been developed based on the results of the field studies reported here, and a greater awareness of the disease should facilitate correct diagnosis and management of CLS in the future.

Appendices

APPENDICES

Appendix 1: List of *Cercospora zonata* isolates used in this study.

Acc. No	Nearest Town
Isolates collected in 2004	
24/04	Saddleworth, SA
36/04	Freeling, SA
37/04	Rhynie, SA
69/04	Turretfield RS DN, SA
70/04	Turretfield RS DN, SA
71/04	Kaniva, Vic
72/04	Kaniva, Vic
73/04	Bool Lagoon S3, SA
74/04	Keith S4, SA
77/04	VS04 Sample 43, SA
Isolates collected in 2005	
19/05	Turretfield RS DN, SA
48/05	Gawler/Freeling, SA
50/05	Gawler/Freeling, SA
52/05	Templers, SA
58/05	Owen, SA
62/05	Artherton, SA
64/05	Maitland, SA
68/05	Sandilands, SA
70/05	Ardrossan, SA
90/05	Turretfield RS, SA
91/05	Keith, SA
92/05	Wolseley, SA
159/05	Dimboola, Vic
160/05	Charlick RS P123 S3
161/05	Charlick RS P49 S3
168/05	Charlick RS, SA
71/05	Glenthorne RS, SA
75/05A	Bool Lagoon, SA
75/05B	Bool Lagoon, SA
178/05	Charlick RS, SA
180/05	Millicent, SA
181/05	Millicent S3, SA
182/05	Bool Lagoon, SA
183/05	Penola, SA
184/05	Millicent, SA
185/05	Bool Lagoon, SA
93/05	Mundulla, SA
Isolates collected in 2006	
29/06	Cummins, SA
32/06	Cockaleechie, SA
37/06	Pathaway, SA
74/06	Bool Lagoon, SA

Acc. No	Nearest Town
Isolates collected in 2007	
PH 5	Koolunga, SA
PH 6	Narridy, SA
PH 8	Saddleworth, SA
PH 11	Hamley Bridge, SA
MF 1	Clare, SA
MF 5	Merilden, SA
MF 7	Watervale, SA
MF 8	Auburn, SA
MF 9	Manoora, SA
MF 10	Riverton, SA
AP 1	Roseworthy, SA
AP 2	Templers, SA
AP 4	Tarlee, SA
AP 6	Tarlee, SA
AP 9	Riverton, SA
AP 10	Saddleworth, SA
YP 1	The Pines, SA
YP 2	Port Turton, SA
YP 7	Minlaton, SA
YP 9	Ardrossan, SA
YP 15	Warooka, SA
PC 1	Crystal Brook, SA
PC 2	Crystal Brook, SA
PC 4	Gladstone, SA
PC 5	Gulnare, SA
PC 7	Gulnare, SA
EP 1	Cummins, SA
EP 3	Cummins, SA
EP 7	Yeelanna, SA
SE 1	Francis, SA
SE 8	Furner, SA
SE 9	Furner, SA
SE 11	Millicent, SA
SE 14	Keith, SA
SE 16	Keith, SA
SE 17	Culburra, SA
VIC 4	Marnoo, Vic
VIC 5	Murtox, Vic
VIC 7	Lubrek, Vic
VIC 11	Longerenong, Vic
VIC 14	Natimuk, Vic
VIC 15	Natimuk, Vic
VIC 17	Nhill, Vic
VIC 19	Yanac, Vic

Acc. No	Nearest Town
Isolates collected in 2008	
79/08	Laura, SA
80/08	Maitland, SA
Isolates collected in 2009	
19/09	Cummins, SA
29/09	Snowtown, SA
36/09	Roseworthy, SA
37/09	She-Oak Log, SA
85/09	Rosedale, SA
86/09	She-Oak Log, SA
87/09	Gomersal, SA
150/09	Bool Lagoon, SA
151/09	Frances, SA
152/09	Millicent, SA
Isolates collected in 2010	
94/10	Bordertown NVT
110/10	Bool Lagoon, SA
111/10	Millicent, SA
112/10	Bordertown, SA

Note:
SA = South Australia
Vic = Victoria
RS = Research Station
DN = Disease Nursery
VS04 = Virus Survey 2004
S3 = Stage 3 breeding trial
S4 = Stage 4 breeding trial
P = Plot
NVT = National Variety Trial

Each isolate was stored in a fungal culture collection maintained by SARDI - Pulse Pathology Laboratory.

Appendix 2*:* Media used for fungal culture

All prepared media were autoclaved at 121°C for 15 min then once cooled to approx 55°C, the agar media were dispensed into 9-cm diameter Petri dishes, approx. 15 ml per plate, and allowed to cool and solidify.

Full Strength Potato Dextrose Agar (PDA)

Potato dextrose agar (Oxoid)	39 g
RO (reverse osmosis) water	1000 ml
1% Streptomycin sulphate (Sigma)	10 ml

Culture medium was autoclaved at 121°C for 20 min and, in a sterile laminar flow unit, 1% streptomycin (1 g/100 ml sterile RO water) was added to molten medium once cooled to approx. 55°C. Agar medium was dispensed as above.

Cornmeal Agar (CMA)

Cornmeal (ground maize)	40 g
RO water	500 ml
Bacto Agar (Oxoid)	10 g

The cornmeal and water were combined, mixed, then placed in a water bath (ca 55°C) for 1 h. The suspension was then filtered through one layer of Whatman (No. 1) filter paper then Bacto agar was added and dissolved by bringing the suspension to boil. The culture medium was autoclaved and dispensed as above.

Bean Seed Agar (BSA) (Foudin AS, 1987. *Mycologia* 79, 117-122)

Finely ground (2 mm) faba bean seed (cv. Fiord)	20 g
RO water	1000 ml
Bacto Agar (Oxoid)	10 g

Bean flour and RO water were autoclaved at 121°C for 60 min then allowed to stand at room temperature for 18 h. A 500 ml suspension was filtered through four layers of cheesecloth, then Bacto agar was added and dissolved by bringing the suspension to boil. The culture medium was autoclaved and dispensed as above.

Carrot Juice Agar (CJA) (Baxter LW, Fagan SG, 1974. *Plant Disease Reporter* 58, 300)

Sliced carrots (*ca* 2 mm thick discs)	20 g
RO water	1000 ml
Bacto Agar (Oxoid)	10 g

Appendix 2: continued

The carrots and RO water were soaked for 1 h then boiled for 5 min. A 500 ml aliquot of the resultant puree was decanted through a flour strainer (1 mm) and Bacto agar was added and dissolved by bringing the suspension to boil. The culture medium was autoclaved and dispensed as above.

Carrot Juice Agar, unfiltered (CJA2)

Boiled carrots (remaining from CJA preparation)	500 ml
Bacto Agar (Oxoid)	10 g

The remaining 500 ml of the unfiltered puree prepared for CJA was used and Bacto agar was added and dissolved by bringing the suspension to boil. The culture medium was autoclaved and dispensed as above.

Carrot Leaf Decoction Agar (CLDA) (Chen MD, Lyda SD, Halliwell RS, 1979. *Phytopathology* 46, 180)

Fresh carrot leaves	200 g
RO water	375 ml

Carrot leaves and RO water were brought to boil, then allowed to simmer for 1 h 45 min. During this time, sterilised molten agar was prepared

RO water	350 ml
Bacto Agar (Oxoid)	10 g

Bacto agar was added to RO water, and dissolved by bringing the suspension to boil. The medium was autoclaved at 121^{o}C for 20 min after which, using aseptic techniques within a laminar flow cabinet, 150 ml of boiled carrot leaf juice was decanted into the molten agar in a 500 ml Schott bottle. The bottle, containing the culture media, with lid was then steamed sterilised within a stainless steel flask of boiling water for 1 hr, but not autoclaved. Once cooled to approx 55^{o}C, the agar medium was dispensed as above.

Carrot Leaf Pulp Agar (CLPA)

Boiled carrot leaves (remaining from CLDA preparation)	300 ml
Bacto Agar (Oxoid)	10 g

The remaining 300 ml of unfiltered carrot leaf juice prepared for CLDA was used and Bacto agar was added and dissolved by bringing the suspension to boil. The culture medium was autoclaved and dispensed as above.

Appendix 2: continued

V8 Agar (V8A) – 20% concentration (Miller PM, 1955. *Phytopathology* 45, 461)

V8® juice (unfiltered)	100 ml
RO water	500 ml
$CaCO_3$	2.3 g
Bacto Agar (Oxoid)	10 g

The V8 juice and water were combined and then $CaCO_3$ added to adjust pH of the suspension to neutral (ca pH 7). Bacto agar was added and dissolved by bringing the suspension to boil. The culture medium was autoclaved and dispensed as above.

V8 Broth – concentration altered as required

V8® juice (unfiltered)	100 ml
RO water	500 ml
$CaCO_3$	2.3 g

The V8 juice and water were combined and then $CaCO_3$ added to adjust pH of the suspension to neutral (*ca* pH 7). The suspension was gently mixed and the culture medium was autoclaved at 121^{o}C for 20 min then once cooled to approx 55^{o}C, the media were dispensed into 9-cm diameter Petri dishes, approx. 15 ml per plate, and allowed to cool.

This recipe is for a 20%V8 broth. Alternative concentrations were prepared by changing the ratio of V8 juice and RO water.

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(Chapters 1 – 4 and 9)

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