STEMPHYLLIUM GREY LEAF SPOT DISEASE OF LUPINS IN WESTERN AUSTRALIA

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Stemphylium Grey Leaf Spot Disease of Lupins in Western Australia

Grey leaf spot lesions on leaflets of narrow-leafed lupin plant
Abstract

One of the traditional principles of disease management is avoidance of the pathogen. This study was planned to investigate the parameters that could be used to develop strategies for avoiding Stemphylium grey leaf spot (GLS) disease of narrow-leafed lupins (NLL) in Western Australia (WA). To achieve this objective, the project was divided into four different components.

Firstly, surveys of WA lupin growing regions were carried out over three years to ascertain the presence and distribution of virulent strains of the pathogen in the field. Secondly, the impact of virulent strains of the pathogen on grain yield of NLL was assessed. These two components were planned to investigate the importance of the disease to the Western Australian NLL crop in terms of distribution and potential impact on production. The third component focused on the environmental requirements of the disease. Temperature and moisture requirements for optimum growth of the pathogen and for establishment of infection were studied in vitro. These optimum requirements were then tested in a glasshouse under controlled conditions. During the fourth component, ability of the pathogen to infect other plants commonly found in WA and its persistence on the infested trash and infected seed was investigated to determine the survival strategy of the pathogen. Finally, a predictive risk model was developed for WA based on the available information.

Across the three survey year, seven virulent isolates of Stemphylium spp. were found. Two of those were highly virulent on susceptible genotypes of NLL whereas five were moderately virulent. One of the highly virulent isolates was collected from the NLL growing in the northern agricultural region of the wheatbelt of WA, while the other was isolated from the lupin breeding shadehouse at Medina. Under favourable conditions in inoculated plots, highly virulent isolates were found to cause up to 60% grain yield loss in susceptible genotypes of NLL.

Morphological and molecular characterisation of the virulent isolates was carried out to ascertain the species of the pathogen involved in the disease. In addition to previously reported *S. botryosum* and *S. vesicarium*, *S. solani* –a new record for WA – was found associate with GLS disease in WA. Disease symptoms on NLL seedlings were similar for all isolates and variety resistance was effective against all isolates.
Under *in vitro* conditions, cyclic day/night temperatures of 25/20 °C were optimum for sporulation and germination of the pathogen whereas 30/25 °C favoured the mycelial growth and germ-tube elongation. At 25/20 °C a minimum leaf wetness period of 6 h was required for infection to occur. Leaf wetness of 12 h resulted in appearance of light infection whereas 24 h wetness promoted severe infection in excised NLL leaves under controlled environment conditions. The results of the *in-vitro* and controlled environment studies were supported by results for spray inoculated seedlings under glasshouse conditions.

Both infested trash and infected seed can carry over inoculum between seasons. Spore trapping and trap plant bioassays showed that the pathogen within infested trash remains infective for at least 12 months and that conidia release and seedling infection are closely linked to rainfall events.

A weather-driven disease risk model was developed based on data collected from the laboratory, controlled environment, glasshouse and spore trapping studies. The model used weather parameters of temperature and rain days per week to predict disease development and therefore the comparative risk of GLS disease across lupin producing regions of WA for three seasons (2010-2012). Infection risk varied between sites and seasons. The region of greatest risk was identified as the western coastal zone between Perth and Geraldton. Strategies to avoid the pathogens associated with GLS or to manage the risk of disease development can be devised as a direct outcome of this research project.
Statement of the contributions

Research papers prepared for publication:

1. A. Ahmad, G.J. Thomas, S.J. Barker and W.J. MacLeod. Morphological and molecular characterization of Stemphylium sp. isolates causing grey leaf spot disease of lupins in Western Australia. Presented in Chapter 3

Contribution:

A. Ahmad: Sample collection, morphological and molecular work, and manuscript preparation

G.J. Thomas: Sample collection and critical revision of the manuscript

S.J. Barker: Primer selection, PCR program design and critical revision of the manuscript

W.J. MacLeod: Critical revision of the manuscript


Contribution:

A. Ahmad: Experiment design, collection and analysis of data, and manuscript preparation

G.J. Thomas: Experiment design and critical revision of the manuscript

S.J. Barker: Critical revision of the manuscript

W.J. MacLeod: Critical revision of the manuscript


Contribution:

A. Ahmad: Experiment design, collection and analysis of data, and manuscript preparation

G.J. Thomas: Experiment design and critical revision of the manuscript
S.J. Barker: Critical revision of the manuscript

W.J. MacLeod: Experiment design

4. Host range of Stemphylium, overwintering of the pathogen on debris and infection of lupin seed. Presented in Chapter 6

**Contribution:**

A. Ahmad: Experiment design, collection and analysis of data, and manuscript preparation

G.J. Thomas: Experiment design, collection of data and critical revision of the manuscript

S.J. Barker: Critical revision of the manuscript

W.J. MacLeod: Experiment design

The contributors agree that the above statement accurately describes their contribution to the research manuscript and give consent to their inclusion in this thesis.

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CHAPTER 1

Introduction

Lupins (*Lupinus* L.), commonly considered garden flowers or green manure plants, are a critical component of the Western Australian agricultural system, the wheat:lupin rotation. Being a legume, lupins fix atmospheric nitrogen via a rhizobium-root nodule symbiosis. Growing lupins in rotation with cereal crops, such as wheat and barley, can reduce the need for nitrogenous fertilizer and boost cereal yields. Adaptability to diverse environments makes lupin a more attractive grain legume crop than many other available legume species. Nitrogen fixation, together with a deep root system and ability to form cluster roots, explains the tolerance of lupins to the infertile soils of Western Australia (WA) (Hamblin and Hamblin, 1985; Hamblin and Tennant, 1987). Lupin production worldwide followed an increasing trend during 1980-1999 with the vast majority of this increase coming from Australia.

Narrow-leafed lupin (*Lupinus angustifolius* L.; NLL) is grown in Australia, Europe, South Africa and South America. This lupin species is a relatively recent commercial crop in Australia. The species was developed as a new crop in Australia by J. S. Gladstones in the 1960s from semi-domesticated *L. angustifolius* introduced from Europe (Cowling and Gladstones, 2000). It has been domesticated for about 50 years, since the first introduction (Gladstones, 1998). Currently the majority of the Australian lupin production is NLL and more than 80% of this crop is grown in WA (French and Buirchell, 2005). Like other legume crops, NLL is also a good source of protein and dietary fibre. NLL seeds are widely used as animal feed and have potential uses for human food consumption (Duranti et al., 2008; Jappe and Vieths, 2010).

Fungal pathogens can be a significant constraint to production of NLL. Thirty years ago the main diseases inhibiting the development of NLL in Europe were Fusarium root rot, Fusarium wilt, Botrytis grey mould, brown spot, and grey leaf spot (GLS) (Kutpsov et al., 2006). Currently in WA some of the most potentially damaging and economically important fungal pathogens of NLL are anthracnose stem and pod blights, caused by *Colletotrichum lupini* (Nirenberg et al., 2002), *Pleiochaeta* root rot and brown leaf spot caused by *Pleiochaeta setosa* (Sweetingham et al., 1998), *Rhizoctonia* bare patch caused by *Rhizoctonia solani* and Phomopsis stem and pod infection caused by
Diaporthe toxica (Thomas et al., 2008). Soon after the domestication of NLL in WA, in the 1970s, GLS disease was a major concern for local NLL growers (Cowling and Gladstones, 2000).

GLS in lupins is caused by Stemphylium spp. The first report of the disease came from the USA in 1956 and the causal organism was identified as S. solani (Wells et al., 1956), but five years later S. botryosum was reported to cause identical symptoms on blue lupins (Wells et al., 1961). Severe damage was observed at some locations in Georgia and Florida, demonstrating that GLS disease seriously reduces forage and seed production of lupins when the environment is favourable for disease development. Both fungi caused extensive defoliation during seed setting and were considered responsible for reduced seed yields in susceptible varieties (Edwardson et al., 1961). In 1962 another species, S. loti, was found to be highly pathogenic on breeding lines of blue lupins that were resistant to the two earlier reported species (Wells et al., 1962).

In 1970s, wild ecotypes of NLL were found that were resistant to the disease, which provided a source of resistance that was later incorporated into the domestic types as part of a collaborative breeding program between WA and the USDA (Forbes et al., 1975; Gladstones, 1994). Resistance to GLS in the Western Australian cultivars Marri (1976) and cv. Illyarrie (1979) was a major breakthrough in the development of the crop (Cowling and Gladstones, 2000). Over the last three decades GLS has not been an issue for WA due to the presence of resistance in the majority of NLL varieties released in that time (Thomas et al., 2008). Recently however, GLS has been again observed at damaging levels in lupin breeding plots (Figure 1.1 and Figure 1.2) and it has become evident that a proportion of the existing breeding material no longer contains resistance to this disease (Thomas et al., 2011). The release of susceptible varieties and possible changes to local environments as a result of climate change could favour the survival and spread of the pathogen making the crop more vulnerable to attack on a large scale. The recent re-emergence of GLS disease (Thomas et al., 2011) prompted the need to undertake studies to further investigate the disease in WA.
Figure 1.1: Grey leaf spot lesions at an advanced stage on leaflets of susceptible narrow-leafed lupin genotype WALAN2333
Figure 1.2: (a) Grey leaf spot lesions on pods of susceptible narrow-leafed lupin genotype WALAN2333, (b) Grey leaf spot lesions on stem of susceptible narrow-leafed lupin genotype WALAN2333
The current study was conducted to achieve the following objectives;

1. To identify the pathogen associated with GLS disease in WA by comparing: the morphological features; growth characteristics; a portion of the DNA sequence of isolates of *Stemphylium* spp. collected from symptomatic tissue on lupins in the WA agricultural region

2. To determine the economic significance of the disease by examining potential yield losses caused by the pathogen

3. To determine environmental conditions favourable for the disease, and the spread and survival of the pathogen

4. To develop an epidemic risk model for lupin producing regions of WA

The thesis is structured as a series of papers, each with an abstract, introduction, materials and methods, results and discussion. As short general discussion is then presented in order to integrate results from all chapters. References from all chapters are collated and presented at the end of the thesis.
CHAPTER 2

Literature Review

2.1 Lupins in Western Australia

Genus *Lupinus* is divided into two major groups: the Mediterranean/north and east African 'Old World' species, and the North and South American 'New World' group. It includes a diverse range of species (Cowling et al., 1998). The Old World group is subdivided into the rough-seeded and the smooth-seeded species. Narrow-leafed lupin (*Lupinus angustifolius* L.; NLL) belongs to the smooth seeded group of species in conjunction with two other cultivated species: *L. albus* and *L. luteus* (Gladstones, 1998).

NLL probably originated in Palestine and diversified in the Aegean region (Clements et al., 2005). The smooth seeded genotypes that originated from Spain were cultivated in northern Europe in the 19th Century. In southwest France and Germany lupin cultivation started in the early 19th century. By the mid-19th Century lupin cultivation was started in Suffolk, England, for sheep feed, and this type of grain legume was heavily relied upon by the Saxony merino wool industry (Gladstones, 1998). *L. angustifolius* which is tolerant to frost, less fertile and more acidic soils, had advantage over *L. albus* and was readily accepted by the European sheep farmers (Gladstones, 1998). By the early 20th century, bitter *L. angustifolius* was grown with *L. luteus* in Russia and the northern Ukraine (Gladstones, 1998). After the turn of the 19th Century, however, lupin cultivation fell, due to the introduction of cheap nitrogen fertilizers, and, in the more advanced economies, rising land values, which favoured continuous non-legume cash crops (Clements et al., 2005).

NLL was introduced to Australia along with New Zealand and South Africa during early 1900s (Gladstones, 1998). In South Africa, New Zealand and the US, the crop was used as green manure and sheep feed (Hudson, 1934; Gladstones, 1970). It was introduced into Australia in the 1930s and 1940s, mainly for soil improvement in orchards and vineyards (Gladstones, 1970). In Australia, a local breeding program based on *L. angustifolius* was initiated by Dr. John Gladstones in the late 1950s (Gladstones, 1998). From zero commercial production just prior to the release of the first Australian cultivar in 1967, there was a steady increase in area under lupin cultivation to a maximum of 1.425 million ha in 1997. Lupins were introduced just at
the end of a period of rapid expansion and clearing of land for agriculture in southwestern Australia. At the time of introduction of lupins, the subterranean clover-wheat rotation system was in decline because of weeds, disease, and reduced pasture seed banks. A new legume with the characteristics of lupin was a welcome solution as a rotation crop. Lupins were also perfectly suitable to the coarse-textured, acidic and infertile soils of the wheat-belt of Western Australia (WA) and this region represents more than 90% of the area sown in Australia (Perry et al., 1998). Area under lupin cultivation expanded successively over three decades. The expansion was achieved through a dedicated breeding and evaluation program (Cowling and Gladstones, 2000). Lupins are now entering a new phase with prospective high-value markets for the grain as a quality feed, including aquaculture and the human food industry, based on several excellent functional properties of the protein and fibre fractions. (Clements et al., 2005; Li, 2012). It will be essential to understand and manage any disease threat to profitability of this emerging market.

2.2 Fungal diseases of lupins

The main diseases that hindered the development of NLL in Europe in 1970’s were Fusarium wilt, brown spot, and grey leaf spot (GLS) (Kutpsov et al., 2006).

Currently in WA, anthracnose caused by Colletotrichum lupini (Nirenberg et al., 2002), brown leaf spot caused by Pleiochaeta setosa (Sweetingham et al., 1998), phomopsis stem and pod infection caused by Diaporthe toxica and Sclerotinia stem rot caused by Sclerotinia sclerotiorum are the major fungal diseases affecting foliage, stems and pods of lupins (Thomas et al., 2008). Anthracnose of lupin was reported first in 1939 on NLL (Sweetingham et al., 1998). It was first detected on commercial lupin crops in WA in 1996 (Sweetingham et al., 1998). The disease is very harmful and represents a substantial threat to cultivation of lupins worldwide. The disease can affect cotyledons, leaves, stems and seed of NLL. The most distinctive symptom is twisting and bending of stems with a lesion in the crook of the bend. Similar distortion of pods can occur. Lesions contain masses of pink-orange conidia which are spread by rain-splash to infect adjacent healthy plants when hit with drops of rain. The fungus is also seed borne (Thomas, 2003; Thomas et al., 2008).

Brown spot (Pleiochaeta setosa) is the most widespread disease of lupins in WA. The pathogen survives as conidia in the soil and infects seedlings in the subsequent growing
season. Infected cotyledons develop dark brown spots and rapidly die and drop off the plant. Leaves also develop dark brown spots that are often net-like in appearance, and symptomatic leaves can be distorted and reduced in size before prematurely dehiscing. The disease can infect lupins at all stages of growth but seedling infection has the greatest impact on yield (Thomas and Coutts, 2009).

Phomopsis is caused by the fungus *Diaporthe toxica*, it is of particular importance to the use of trash and seed for animal feed as the fungus produces a mycotoxin when it grows in senescing stems. The toxin causes lupinosis in livestock grazing the infected stubble, affected animals can become sick or die. Symptoms of phomopsis infection appear as lesions containing black fruiting bodies on dry stems, pods and trash at the end of the growing season. The fungus infects the growing lupin plant but usually remains symptomless until plants reach physiological maturity. If lesions develop on pods prior to harvest, seed can become infected and will show gold or brown discolouration (Thomas et al., 2008).

*Sclerotinia stem rot* (*Sclerotinia sclerotiorum*) infects most broad leaf crop and pasture species. It is most common in higher rainfall areas and usually affects plants after flowering in warm and damp conditions. The fungus produces a white cottony-looking growth that girdles the stem, causing the plant parts above the lesion to wilt and die (Thomas and Coutts, 2009).

### 2.3 Grey leaf spot disease of lupins

**2.3.1 History of the disease**

The first report of GLS disease came from the USA in 1956 and the causal organism was identified as *S. solani* (Wells et al., 1956) but five years later *S. botryosum* was reported to cause identical symptoms on blue lupins (Wells et al., 1961). Severe damage was observed at some locations in Georgia and Florida, demonstrating that the GLS seriously reduces the forage and seed production when the environment is favourable for disease development. Both fungi caused extensive defoliation during seed setting and were considered responsible for reduced seed yields in susceptible varieties (Edwardson et al., 1961). In 1962 another species *S. loti* was found to be highly pathogenic on breeding lines of blue lupins that were resistant to the two earlier reported species (Wells et al., 1962). In New Zealand, GLS disease causing foliar
lesions and defoliation of lupins was attributed to infection by *S. botryosum* (Tate, 1970).

GLS disease was reported to cause significant damage and yield losses to lupin crops in WA in the early 1970s and the causal fungus was reported to be *S. vesicarium* (Gladstones, 1977). It became recognised as a one of the major diseases of NLL at that time and breeding efforts were directed at incorporating resistance to the disease (Cowling and Gladstones, 2000). Resistance to GLS was transferred from US breeding lines to Australian NLL genotypes through collaboration with the US Department of Agriculture. Resistance in cultivars Marri (1976) and Illyarrie (1979) was derived from US cultivar Rancher (Gladstones, 1982), which contained resistance genes sourced from a Portuguese wild type (Forbes et al., 1965).

After the successful incorporation of GLS resistance into local Australian varieties, the disease was no longer a problem for WA lupin industry until recently, when it was again observed to severely damage plants in lupin breeding plots. It has been shown that the majority of cultivars released since the 1970s have been resistant to GLS but that a significant proportion of the more recent breeding population no longer contains resistance to this disease (Thomas et al., 2011). As a result, screening varieties for resistance has recommenced in the Western Australian lupin breeding program.

### 2.3.2 Symptoms description

It is reported that diagnosis of the disease can sometimes be complicated because of occurrence of the disease in complex with brown spot and stem canker (Tate, 1970).

Descriptions of symptoms are relatively consistent from disease occurrences in several different environments across the world. Initial infection by the pathogen produces numerous fudge coloured circular lesions on leaflets, ranging in size from a pinpoint up to a millimetre in diameter (Wells et al., 1956). However, advanced lesions grow up to 7 mm in diameter (Tate, 1970). Mature leaflets or seedling cotyledons exhibit circular or semi-circular greyish lesions with ash coloured centre (Figure 1.1). Immature lesions lack the lighter coloured centre. Pod or stem lesions initially appear circular, superficial and grey brown in colour surrounded by a grey halo but mature lesions are more penetrating, with a concave surface, no halo and up to 5 mm in diameter (Figure 1.2). Stem splitting may occur at a lesion under severe infection (Tate, 1970; Thomas et al., 2011).
2.3.3 Causal fungus

Four species of *Stemphylium* (*S. solani*, *S. botryosum*, *S. loti* and *S. vesicarium*) have been reported to cause GLS disease in lupins across various parts of the world where lupins are cultivated (Wells et al., 1956; Edwardson et al., 1961; Wells et al., 1961; 1962; Wells et al., 1971; Gladstones, 1977). However, in WA only two species of the fungus (*S. vesicarium* and *S. botryosum*) have been reported associated with the disease (Gladstones, 1977; Thomas et al., 2011). Disease symptoms associated with various *Stemphylium* species appear to similar (Wells et al., 1956; Wells et al., 1961; 1962; Tate, 1970; Thomas et al., 2011).

2.3.4 Biology of *Stemphylium* spp.

The asexual stage or anamorph of the pathogen is *Stemphylium* while the sexual stage or teleomorph is known as *Pleospora* (Simmons, 1969). Genus *Pleospora*, belongs to the kingdom Fungi, phylum Ascomycota, class Ascomycetes, order Pleosporales, family Pleosporaceae, but the asexual stage or anamorph *Stemphylium* is more commonly known (Ellis, 1971). More than 33 species are recognized in this genus (Camara et al., 2002). Two new species have been recently characterized (Wang et al., 2010). Many *Stemphylium* species are saprophytic, growing on dead plants and cellulose materials (Simmons, 1969; Ellis, 1971), but several species, including *S. botryosum*, *S. solani* and *S. vesicarium* are plant pathogens that cause diseases on important agricultural crops and fruit trees (Wang et al., 2010).

2.3.4.1 Microscopic morphology of *Stemphylium* spp.

Conidiophores are short, aseptate, swollen at the apex, and may be single or in a group. Conidiophores proliferate further after a conidium is produced, producing new cells and new conidia (Simmons, 1967).

Conidia are olive brown, oblong or muriform in shape with three constricted transverse septa (Ellis, 1971). The size of mature conidia varies from 13 to 78 µm in length and 7 to 24 µm in width, whereas the size of conidiophores varies from 25 to 312 µm in length and 2 to 6 µm in width in different species of *Stemphylium* (Camara et al., 2002). Ascospores (32-48× 12-21 µm) are elongate to oval, with seven horizontal and three to five longitudinal septa, and yellowish to brown in colour and appear muriform on maturity. Perithecia are globose, membranous and black and sometimes have a slender
neck. Asci (183-267× 27-37 µm) are oblong to clavate with distinct outer and inner walls (Bayaa and Erskine, 1998).

2.3.4.2 Colony characteristics

*Stemphylium* colonies grow rapidly on a variety of media. On most media, the colonies are velvety to cottony in texture with a grey, brown or brownish-black colour and black pigmentation on the colony reverse (Mwakutuya, 2006). Conidial density is low in cultures produced under laboratory conditions even when the isolate is grown under alternate cycle of 12 h light and 12 h darkness on potato dextrose agar medium (Bashi and Rotem, 1975b).

2.3.4.3 Taxonomy

Various attempts have been made to establish criteria for taxonomy of *Stemphylium*. Hughes (1953) attempted to establish the fundamental principles of differentiation. However, Simmons (1967) was pivotal in recognizing differences in conidiophore organization and shape of conidiogenous cells as the principal morphological characteristics that clearly distinguish *Stemphylium* from two similar genera, *Ulocladium* and *Alternaria*. Simmons (1969) established criteria to differentiate among various species of *Stemphylium* and assigned *P. herbarum* as teleomorph of *S. botryosum*. However, Simmons (1985) subsequently reclassified *Stemphylium/Pleospora* holomorphs and reported that *S. botryosum* and *P. herbarum* were not components of the same holomorph. *P. tarda* was assigned as the teleomorph of *S. botryosum* and *S. herbarum* as the anamorph of *P. herbarum*.

Molecular characterization has been attempted in the taxonomy of *Stemphylium*. Phylogenetic study of the relationship among the species and isolates of the genus *Stemphylium* revealed a monophyletic origin (Camara et al., 2002). Another phylogenetic study revealed that *Alternaria* and *Ulocladium* species clustered within a large monophyletic clade with *Stemphylium* as the sister taxon (Pryor and Bigelow, 2003).

Camara et al. (2002) studied the phylogenetic relationships of 43 isolates representing 16 species of *Stemphylium* using ITS and glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene sequence data. The morphological species classification was supported by the molecular results. Species pathogenic to alfalfa were separated into two groups. *S.*
botryosum and two isolates with morphological characters similar to S. globuliferum had identical sequences at both loci. These two loci in S. vesicarium, S. alfalfae and S. herbarum were almost identical but differed from S. botryosum.

Inderbitzin et al. (2009) performed phylogenetic analysis of Stemphylium isolates with particular emphasis on species differentiation in the P. herbarum clade. Cultures of five species including P. herbarum were found inseparable. Being self-fertile the clade was speculated to represent a group of highly inbred, morphologically distinct lineages that have not yet accumulated detectable species-specific sequence variation.

Morphological features such as size and shape of the conidia, conidiophores and ascospores and the size and time of maturation of pseudothecia are used to characterize different species of Stemphylium (Simmons, 1969; Ellis, 1971; Camara et al., 2002). However, other morphological features such as septum development and small variations in conidial wall ornamentation are not considered reliable (Camara et al., 2002).

Different species of Stemphylium are sometimes found to cause identical symptoms in various hosts despite their genetic differences. Chaisrisook et al. (1995) noted at least five genetically distinct species of Stemphylium, causing leaf spot of alfalfa. Similarly, four different species of Stemphylium are reported to cause GLS disease in lupins. The difference between the species may or may not have any bearing on the development and symptom of the disease; an issue that is still to be investigated in the case of GLS of NLL.

2.3.4.4 Infection process

Pathogenic Stemphylium spp. survive on debris of the different host plants that have been studied. After the overwintering period, sexual fruiting structures of Pleospora are produced on the debris (Johnson, 1990; Prados-Ligero et al., 1998; Basallote-Ureba et al., 1999; Kohl et al., 2009). With the onset of favourable conditions, the sexual fruiting structures rupture and discharge ascospores (Falloon et al., 1987) and conidia are also produced from the debris (Johnson, 1990).

Airborne conidia or ascospores of the pathogen land on host tissue and germinate when conditions are favourable to initiate a new generation of infection (Prados-Ligero et al., 2003; Kohl et al., 2009). After germination, penetration of leaves may occur directly
through the epidermis, but under favourable environment conditions, stomatal penetration is the preferable mode of penetration (Suheri and Price, 2000). Stomatal penetration is governed by environmental factors but is also affected by host resistance, as reported for *S. botryosum* by Cowling and Gilchrist (1982b). The hyphae of the fungus enter the cell and spread inside, deriving water and nutrients from the surrounding cells. Eventually these cells die causing tissue to turn brown and appear blighted. Small tan coloured necrotic spots gradually enlarge in size and eventually cover the whole leaf. *Stemphylium* also produces the toxin stemphol that may aid host infection (Solfrizzo et al., 1994).

### 2.3.4.5 Races of the pathogen

Existence of different races of *Stemphylium* is not commonly known. However, variation in virulence of different races of *S. solani* and *S. vesicarium* has been reported in tomato (Hernandez, 1985) and alfalfa (Irwin and Bray, 1991), respectively. In asparagus, variation in virulence of *S. vesicarium* races was observed (Bansal et al., 1992). In another study with alfalfa, no race structure was described, but biotypes of *S. botryosum* were reported on the basis of adaptation to cool (18-20 °C) or warm weather (23-27 °C) (Cowling et al., 1981). Differences in relative virulence of *S. botryosum* isolates were correlated with the frequency of stomatal penetration in alfalfa. Isolates with high relative virulence produced a higher proportion of effective stomatal penetrations compared to isolates with low virulence (Cowling et al., 1981).

### 2.3.5 Host range

*Stemphylium* is pathogenic to a wide range of crops in varying geographic regions under diverse environmental conditions. Some of the host crops include cotton (Mehta, 1998), lucerne (Irwin and Bray, 1991), garlic (Boiteux et al., 1994), pear (Kohl et al., 2009), asparagus (Lacy, 1982), lentil (Taylor et al., 2007), spinach (Koike et al., 2005), tomato (Bashi et al., 1973), alfalfa (Cowling et al., 1981), clover (Graham, 1957) and lupin (Gladstones, 1994). The ability of pathogens to infect a wide range of crops reflects its adaptability to wide range of climatic conditions and provides better survival chances.
2.3.6 Epidemiology of Stemphylium

2.3.6.1 Survival

Based on the studies on other crops on which *S. botryosum* is pathogenic, the fungus survives between cropping cycles on infected plant debris, on seed and in the soil.

Plant debris is considered the primary source of inoculum of *Stemphylium* in most host-plant species. In garlic, *P. herbarum* overwinters on diseased plant debris that provides primary source of inoculum in subsequent leaf spot epidemics (Basallote-Ureba et al., 1999). On alfalfa, *S. botryosum* has been reported to overwinter on dead stems and leaves (Flint and Clark, 1981). In asparagus, ascospores from overwintering pseudothecia act as source of inoculum for infection of spears (Elmer et al., 1996). Infection of *S. vesicarium* on pear increased every year due to accumulation of inoculum in plant debris (Polfliet, 2002). In lupins diseased plant debris is considered to play an important role in the early infection by *Stemphylium* (Thomas et al., 2011).

Very few studies have determined the duration and conditions for survival of the pathogen on plant debris which may be quite variable depending on the host-plant species. The duration of viability of the pathogen on debris is of prime concern especially in WA farming system where cultivation of lupins is primarily as a rotation legume. In some hosts burial of debris proves effective disease management strategy by avoiding infection at early stages of seedling development. Srivastava et al. (1996) reported that *S. vesicarium* remained viable for 4 months on diseased plant debris, for 3 months at soil depths of 2.5, 5.0 and 7.5 cm and for 2 months at soil depths of 10 and 15 cm. The burial of the infected plant debris thus reduced the disease severity in the coming season. Similarly, burial of spinach debris reduces viability of the ascospores over time as compared to surface debris. Although burial of the debris reduces the viability of ascospores and their subsequent release it does not fully prevent pseudothecial maturation (Prados-Ligero et al., 1998). However, burial of asparagus debris has been observed to fully prevent pseudothecial maturation and subsequent ascospore release (Johnson, 1990). This suggests that residue burial is a vital component of integrated control of the diseases caused by *Stemphylium*.

Infected seed is an important means of transmission of plant diseases and also serves as a source of initial inoculum early in the season. Hernandez-Perez (2006) reported that *S. botryosum* causes internal infection on spinach seed. Based on the results of a
component seed assay, the pathogen was detected on 54% of the pericarps and 29% of the embryos. The internal infection rendered seed treatments ineffective. However, in onions fungicide use was found to be effective against seed-borne *Stemphylium* (Aveling et al., 1993). There is a need to determine the importance of NLL seed as a source of *Stemphylium* infection, for both routine crop protection and national and international biosecurity.

### 2.3.6.2 Within crop spread of the pathogen

Spread of the pathogen is through airborne conidia produced on the surface of infected tissue during the season in garlic and leeks (Suheri and Price, 2001; Prados-Ligero et al., 2003). However, under orchard conditions, conidia are not originated from infected leaves or fruits in pears infected by *S. vesicarium* (Rossi et al., 2005). Thus the disease cycle of the pathogen varies for different hosts. The inoculum may only be produced during the season from infested debris as in pears or from the newly infected tissues of the host during the season (Falloon et al., 1987; Basallote-Ureba et al., 1999; Rossi et al., 2005).

As with other pathogens, ascospores of *Pleospora* are discharged into the air from the protruding pseudothecia that act as source of primary inoculum (Prados-Ligero et al., 2003). In asparagus, both conidia and ascospores of *P. herbarum* on debris from the previous year's fern growth serve as primary inoculum. Infected asparagus seedlings growing as weed during the harvest season of spinach act as a bridge to carry inoculum of *S. vesicarium* from one season to another (Johnson, 1990). Environmental factors have been found to affect the spread and release of spores of *Stemphylium*. Studies on *S. vesicarium* on garlic revealed that rainfall was directly related to the release of spores from debris during the cropping season Relative humidity played an important role in the absence of rainfall (Prados-Ligero et al., 2003).

### 2.3.6.3 Factors affecting disease development

Environmental factors are of prime importance as they affect the initiation and development of plant disease by affecting the host-pathogen interaction (Campbell and Madden, 1990; Agrios, 2005). Temperature and moisture are the most critical environmental variables in the epidemiology of most fungal diseases. Species of *Stemphylium* seem to occur over a wide range of environmental conditions (Basallote-Ureba et al., 1999; Suheri and Price, 2000).
Environmental conditions play an important role in incidence of *S. vesicarium* in pear (Boshuizen et al., 2004). The importance of temperature and moisture for successful development of *Stemphylium* blight in lentil was elaborated through surveys and field experiments in India and Bangladesh (Bakr, 1993; Sinha and Singh, 1993). These variables act as a major driving force behind all the phases of the disease cycle. Presence of moisture on leaf surface is a critical factor for the development of most foliar fungal pathogens (Huber and Gillespie, 1992). Temperature and moisture are primary environmental factors affecting mycelial growth, sporulation and conidial germination of *Stemphylium*, and development of disease on various hosts. Excessive vegetative growth of host plants in combination with abundant moisture favours disease development (Bashi and Rotem, 1974; Emery and English, 1994; Basallote-Ureba et al., 1999; Kim et al., 2004; Mwakutuya and Banniza, 2010).

### 2.3.6.3.1 Temperature

Temperature by itself is the most important environmental variable as it has an effect on almost all biological components of a pathosystem (Campbell and Madden, 1990). At optimum temperature, development of the disease would be rapid, because it influences the rate of conidial germination, germ tube elongation and the time required for host penetration (Agrios, 2005).

In New Zealand, *Stemphylium* infection levels on asparagus were significantly higher at 14 °C than at 20 or 26 °C (Menzies et al., 1991). Sinha and Singh (1993) reported that an average mean temperature of 18±2 °C in the morning was favourable for the infection, development and spread of *Stemphylium* blight of lentil in India. In Bangladesh, *S. botryosum* initiated infection on lentil when the night temperature remained above 8 °C with average day temperature around 22 °C and the relative humidity in the plant canopy exceeded 95% (Bakr, 1993).

Temperature has a direct influence on the survival and spread of the pathogen. In *S. vesicarium*, a positive relationship was reported between conidial concentration in the air and the number of hours with temperatures in the range of 12-21 °C (Prados-Ligero et al., 2003). A significant correlation between number of spores of *S. vesicarium* and days with air temperature between 15 and 25 degrees °C was reported by Rossi et al. (2005) in pear orchards. A study in onion showed that germination of conidia of *S. vesicarium* started within 2 h when incubated at 4 °C (Suheri and Price, 2000). High
Temperature favoured the germination of conidia of *S. botryosum* and under controlled conditions the optimum temperature for conidial germination was between 25 °C and 30 °C (Mwakutuya, 2006). Similar results have been reported from *A. linicola*, where the minimum, optimum and maximum temperatures were 3, 25 and 35 °C respectively (Vloutoglou et al., 1996). Although *A. helianthi* has the same optimum temperature of 25 °C; the spores did not germinate at 5 and 35 °C (Somasundara and Anilkumar, 1987).

Temperature affects the latent period for *S. botryosum* in lentil. At optimum temperature of 25-30 °C, the latent period was minimum (48 h) under controlled conditions. The latent period increased with decrease in temperature and wetness period. The pathogen continued to develop despite interruptive dry periods. Infection units increased after 2 h of incubation at a temperature above 10 °C and the number of infection units was increasingly enhanced with increasing wetting period up to 48 h and increasing temperature up to 30 °C. Optimal conditions for infection were temperatures of more than 25 °C combined with minimum 8 h of wetness (Mwakutuya and Banniza, 2010).

The temperature requirements for optimum disease development seem to vary among populations of *S. botryosum* and from region to region. In the USA, *S. botryosum* causing *Stemphylium* leaf spot of alfalfa was divided into two biotypes differing in their optimum requirements. Cool temperatures (18 to 20 °C) favoured the C-T biotype – mainly a problem in spring and fall in California – whereas warm temperatures (23 to 27 °C), favoured the W-T biotype – mainly prevalent in eastern USA (Cowling et al., 1981). In surveys conducted in Spain, leaf spot outbreaks caused by *S. vesicarium* were favoured by humid conditions followed by dry, warm weather (Basallote-Ureba et al., 1999).

These studies show a combination of environmental factors are important for infectivity of *Stemphylium* conidia. The relationship between temperature and GLS of NLL in WA has not been studied, however these examples from the literature indicate that this factor will be important for determining disease risk.

### 2.3.6.3.2 Moisture

The role of moisture in the initiation and development of infection by *Stemphylium* has been studied in various hosts (Johnson and Lunden, 1986; Montesinos and Vilardell, 1992; Montesinos et al., 1995; Basallote-Ureba et al., 1999; Suheri and Price, 2001;
Splashing rain and running water promote the spread of most plant diseases. *Stemphylium* follows this common theme; the availability of moisture is important for successful infection by and development of this species. Rain, irrigation water, high relative humidity or dew can provide the necessary source of moisture. Most plant pathogens require moisture for spore germination and penetration of the host by the germ tube (Agrios, 2005). The availability of long periods of moisture is important for infection of most host species by *Stemphylium*. Longer wetness periods are associated with increased sporulation, infection and high disease severity by *Stemphylium* (Bradley et al., 2003). The moisture requirements can be quite specific, as demonstrated for the appearance of *Stemphylium* leaf spot in alfalfa (Emery and English, 1994). A strong correlation was observed between the amount of rainfall and the aerial concentration of spores of *S. vesicarium* on garlic (Basallote et al., 1993). In the presence of abundant moisture, 76% conidia of *S. vesicarium* germinated after 32 h (Jakhar et al., 1996). Bradley et al. (2003) recorded 92% germination of conidia of *Stemphylium* after 24 h under continuously wet conditions.

*S. botryosum* on lentil required more than 90% relative humidity and prolonged periods of leaf wetness (Bakr, 1993; Mwakutuya and Banniza, 2010). *Stemphylium* was reported to require at least 8 h of leaf wetness for infection. The infection of onion leaves by *S. vesicarium* occurred after 16 hours of leaf wetness at 5 °C and 8 h of leaf wetness at 10 to 25 °C. Infection increased with increasing leaf wetness duration to 24 h (Suheri and Price, 2000). Jakhar et al. (1996) reported that *S. vesicarium* required at least 16 h in a saturated atmosphere for initiation of infection on onion.

*S. vesicarium* causes severe foliage damage of garlic with subsequent yield loss, only when leaf-wetness periods exceed 24 continuous hours and that warm, humid summers are conducive to development of severe epidemics (Aveling and Naude, 1992). In case of less rainfall, high RH compensates for the required moisture level. High RH during dry periods enables the conidia of *S. vesicarium* to continue germ tube development. In lentil, the number of cloudy and foggy days during the cropping season determine the appearance, development and spread of Stemphylium blight (Sinha and Singh, 1993).

The ability of the conidia to survive dry periods depends on the species and host-pathogen interaction (Llorente and Montesinos, 2002). A dry period of 3 h during a 12 h wet period was adequate to prevent subsequent infection of pear (*Pyrus communis* L.)
by *S. vesicarium* (Llorente and Montesinos, 2002). However, leaf spot outbreaks of garlic and onion caused by *S. vesicarium* were favoured by humid conditions followed by dry, warm weather (Basallote-Ureba et al., 1999). Garlic leaf spot severity was high under foggy and rainy weather in spring, followed by dry warm days (Basallote et al., 1993).

The differences observed between hosts indicate that the interaction of moisture and disease will be important to examine for risk assessment of GLS on NLL in WA.

2.3.6.3.3 **Light**

Various processes in disease development including pathogen sporulation and germination are influenced by light (Agrios, 2005). Fungi exhibit different responses to light, depending on the light intensity, quality, and duration of exposure as well as temperature (Alam et al., 2001). *Stemphylium* is a diurnal sporulator; it requires an alternating light and dark cycle for spore development. In total darkness, it produces only a few spores and sterile conidiophores are formed under constant light (Leach, 1968). Diurnal photoperiod pattern is vital for initiation of sporulation. It was suggested by Leach (1968) that there are two distinct phases of spore formation in *S. botryosum*. The inductive phase – that results in production of conidiophores – is stimulated by light whereas the terminal phase, which initiates the formation of conidia, is inhibited by light. Continuous light inhibited the sporulation of *S. solani* and sterile conidiophores were formed. However, Bashi and Rotem (1975a) reported maximum sporulation in *S. botryosum* on tomato leaves under 24 h light. The release of *S. botryosum* spores borne singly or in chains on simple conidiophores is favoured by low relative humidity in the presence of light (Leach, 1971).

The timing and duration of light exposure influenced the germination of *A. linicola* in the presence of wet leaf surfaces (Vloutoglou et al., 1996). The conidia of *A. solani* germinated in darkness at 25 °C and more than 96% relative humidity (Stevenson and Pennypacker, 1988). The effect of light is obviously variable depending on the pathogen, stimulating spore germination in some fungi and inhibitory in others. Development of Stemphylium blight on lentil is favoured by an average of 7.7 or less hours of sunshine. Eight or more hours of sunshine per day were not favourable for the disease development (Sinha and Singh, 1993). However, the influence of sunshine on disease development may be an indirect flow-on from the effect on relative humidity.
CHAPTER 2

2.3.7 Disease models for *Stemphylium*

Risk of a plant disease is determined by the pathosystem characteristics and environmental conditions. Mathematical and statistical plant disease models are used to understand the role of these factors. The aim of plant disease models is to develop sustainable and effective measures for strategic and tactical management of the diseases. The first of such models was developed by Van der Plank (1960) using a logistic equation to describe the progress of plant disease. Since then, Van der Plank’s equation formed a basis for plant disease modelling. In recent decades, with advancement in technologies and increasing knowledge of epidemiology, more comprehensive models have been developed for many destructive plant diseases.

A model can be defined as a simplification of reality. Models try to represent reality (De Wit, 1993) but they can’t encompass all of the properties of real-life situations (Teng, 1985). Plant disease models summarize main epidemiological processes, to put forward hypotheses and to verify their consistency and consequences (van Maanen and Xu, 2003). A plant disease model is often referred to as a simplification of the relationships between a pathogen, a host plant, and the environmental factors that lead to the epidemic (Rossi et al., 2010).

Disease models for *Stemphylium* spp. have been produced for various host plant species. Montesinos and Vilardell (1992) developed the FAST model for forecasting of early blight on tomato caused by *Stemphylium vesicarium*. Influence of leaf wetness duration and temperature on mycelial growth was studied *in-vitro*. These data were then compared with the weather parameters during epidemic development to produce the FAST model.

Mwakutuya and Banniza (2010) developed a simple logistic model with linear temperature, leaf wetness period, and cross factor effects to describe progression of *Stemphylium* blight of lentil. Germination of conidia, appressorium formation, and infection of lentil plants were evaluated under controlled conditions at temperatures from 5 to 30°C and increasing incubation periods under wet conditions to elucidate the epidemiology of this disease. These data were used to produce the simple logistic equation for disease development.

Prados-Ligero et al. (1998) observed that the pseudothecia of *Pleospora allii* developed best on garlic leaf debris infected by *Stemphylium vesicarium* incubated at low
temperature (5-10 degrees C) and relative humidity (RH) close to saturation. RH of less than 96% prevented the formation of pseudothecia, while an incubation temperature of 15-20 degrees C led to the early degeneration of pseudothecia. Under natural conditions, colonization by pseudothecia of unburied garlic leaf debris varied between seasons from 6.0 to 15.5 pseudothecia/mm(2), whereas lower colonization levels were recorded when samples were buried. Pseudothecial maturity was reached 1-4 mo after the deposition of garlic debris on the soil surface and 15 days after the burial of residues. In the latter case, pseudothecia degenerated with degradation of the plant debris. Ascospore release, which required rainfall or dew periods, occurred between late January and late April depending upon the year. A high correlation was found between pseudothecia maturation and the number of hours with RH greater than or equal to 98% and with a mean temperature of 4.5-10.5 degrees C, and the accumulated rainfall. A multiple regression equation relating the pseudothecia maturity index with these two variables was used to forecast the epidemic onset of Stemphylium leaf spots in Southern Spain.

Boshuizen et al. (2004) modelled Stemphylium vesicarium on pear based on southern European research results. An hourly infection model was prepared based on an infection table from the research data. The model was upgraded subsequent to analysis of field trials, to include consideration of interrupted wetness periods.

A model to predict the risk of Stemphylium grey leaf spot (GLS) disease of NLL has not been developed so far in Australia or elsewhere. This is of increasing importance as lines within the national NLL breeding program lack GLS resistance.
CHAPTER 3

Morphological and molecular characterization of *Stemphylium* spp. isolates causing grey leaf spot disease of lupins in Western Australia

Abstract

Various species of *Stemphylium* cause grey leaf spot (GLS) disease in *Lupinus angustifolius* L. This study was undertaken to investigate the fungal species causing the disease in Western Australia (WA). Surveys of the lupin growing areas of WA were done in 2009-2011 to investigate the occurrence of *Stemphylium* spp. Virulence of the collected isolates and historical isolates from the Western Australian Culture Collection (WAC) was tested on known resistant and susceptible genotypes of narrow-leafed lupin (NLL). Morphological characterization of the isolates was based on length/width ratio, shape and colour of the conidia. Identification of *Stemphylium* to the species level was confirmed by using sequences of the internal transcribed spacer (ITS) of the ribosomal DNA. A total of 66 samples exhibiting leaf spotting were collected during the surveys, 14 of those produced *Stemphylium* isolates. Seven isolates were found to cause varying degrees of disease severity but the variation couldn’t be attributed to differences in species identity. Four different species of *Stemphylium* were identified as being virulent isolates from this survey. The species identified were *S. botryosum* (*Pleospora tarda*), *S. vesicarium*, *S. solani* and *Stemphylium* sp. This study constitutes the first report of *S. solani* associated with GLS disease of NLL in WA.

3.1 Introduction

Cultivation of lupin as a low alkaloid grain crop expanded in Australia after the 1950’s with Western Australia (WA) being the major contributor to production of narrow-leafed lupin (*Lupinus angustifolius* L.; NLL). In early stages of the development and domestication of lupin as a grain crop, Australian varieties were found vulnerable to *Stemphylium* grey leaf spot (GLS) –a disease that caused significant yield losses during the 1970’s (Gladstones, 1977). Resistance to the disease was incorporated into domestic genotypes from exotic sources in a collaborative breeding program with the USDA soon after the initial outbreaks in WA (Gladstones, 1982). This rendered the major genotypes resistant to the pathogen, and the disease has not posed a major risk to WA lupin
production since that time. Recently, however, symptoms of GLS disease were again observed in lupin breeding plots of the Department of Agriculture and Food WA (DAFWA). The re-emergence of this potentially damaging disease prompted the need for further research on the causal agent and its occurrence across WA lupin growing regions (Thomas et al., 2011).

Various species of *Stemphylium* have been reported associated with GLS disease across different countries (Wells et al., 1962; Tate, 1970; Gladstones, 1977; Thomas et al., 2011). As in other parts of the world, *S. botryosum* has been associated with this disease in WA (Thomas et al., 2011) however, WA is the only region to report the association of *S. vesicarium* with the disease (Gladstones, 1977). Genus *Stemphylium* was proposed by Wallroth in 1833 with *S. botryosum* Wallr. as type species and *Pleospora* as anamorph. In excess of 30 species have been recognized in this genus (Camara et al., 2002) most of which are saprophytes growing on dead plants and cellulose materials (Simmons, 1969; Ellis, 1971). However, some species including *S. botryosum*, *S. vesicarium* and *S. solani* are plant pathogens that cause diseases in crops and fruit trees (Bashi and Rotem, 1974; Emery and English, 1994; Basallote-Ureba et al., 1999; Kim et al., 2004; Mwakutuya and Banniza, 2010).

Various efforts have been made to establish the correct criteria for differentiation among different species of *Stemphylium*. Hughes (1953) attempted to establish the fundamental principles of differentiation. However, Simmons (1967) was pivotal in recognizing differences in conidiophore organization and shape of conidiogenous cells as the principal morphological characteristics that clearly distinguish *Stemphylium* from two similar genera, *Ulocladium* and *Alternaria*. Simmons (1969) established criteria to differentiate among various species of *Stemphylium* and assigned *P. herbarum* as teleomorph of *S. botryosum*. However, Simmons (1985) subsequently reclassified *Stemphylium/Pleospora* holomorphs and *P. tarda* was assigned as the teleomorph of *S. botryosum* and *S. herbarum* as the anamorph of *P. herbarum*. Shape, size, colour and length/width ratio of conidia are considered to be the principle morphological characteristics that differentiate the various species of *Stemphylium* (Simmons, 1969; 1985).

Although the identification of *Stemphylium* species is based principally on morphological characteristics of conidia and conidiophores, many of these characters
can overlap among species. As well, conidial characteristics of some fungal species may vary on different substrates and at different temperatures (Leach and Aragaki, 1970). Irwin et al. (1986) used length/width ratio of conidia and other morphological characteristics to identify three species of Stemphylium from Australian isolates. They reported the presence of a group intermediate between S. botryosum and S. vesicarium, however, they included that group in S. vesicarium due to its closeness to the species compared to S. botryosum. Thus correct determination of species based on morphological attributes is often difficult. DNA sequence data are now commonly being used to test the morphological concept and other taxonomic hypotheses (Hunter et al., 2006).

Molecular characterization has been attempted in the taxonomy of Stemphylium. Phylogenetic study of the relationship among the species and isolates of the genus Stemphylium revealed a monophyletic origin (Camara et al., 2002). Another phylogenetic study revealed that Alternaria and Ulocladium species clustered within a large monophyletic clade with Stemphylium as the sister taxon (Pryor and Bigelow, 2003). Inderbitzin et al. (2009) performed phylogenetic analysis of Stemphylium isolates with particular emphasis on species differentiation in the P. herbarum clade. Being self-fertile the clade was speculated to represent a group of highly inbred, morphologically distinct lineages that have not yet accumulated detectable species-specific sequence variation.

The capacity to base the identification of Stemphylium on molecular phylogenetic as well as morphological analyses will improve the accuracy of pathology determinations.

The objective of this investigation was to survey the WA lupin growing regions for the occurrence of Stemphylium sp. infecting lupins, identify and characterize the collected isolates of Stemphylium using morphological and ITS rDNA sequences and compare those with historical isolates of the fungus. Further, by testing their virulence against a set of previously established susceptible and resistant genotypes of NLL the objective was to ascertain the current virulence spectrum of disease to better inform the national NLL breeding program and as well, the international lupin breeding programs.
3.2 Materials and methods

3.2.1 Culture origins of Stemphylium isolates

*Stemphylium* isolates from lupins in WA were obtained from plants exhibiting leaf lesions similar to GLS disease from a wide range of locations in 2009-2011. Samples of leaflets with lesions were collected from NLL plants growing in crops or from known susceptible varieties in crop variety trials or from sandplain blue lupins (*L. cosentenii*) growing wild adjacent to farmer fields. Historical isolates from WA, plus a single isolate from South Australia (SA), were obtained from the Western Australian Culture Collection (Table 3.1).

**Table 3.1: Historical isolates of Stemphylium from lupins included in the analysis**

<table>
<thead>
<tr>
<th>Accession</th>
<th>Year</th>
<th>Pathogen&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Host</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>WAC269</td>
<td>1966</td>
<td><em>Stemphylium sp.</em></td>
<td>Lupin</td>
<td>Dandaragan (WA)</td>
</tr>
<tr>
<td>WAC3719</td>
<td>1982</td>
<td><em>S. vesicarium</em></td>
<td>Lupin</td>
<td>Mingenew (WA)</td>
</tr>
<tr>
<td>WAC5462</td>
<td>1988</td>
<td><em>Stemphylium sp.</em></td>
<td>narrow-leaved lupin</td>
<td>Unknown (WA)</td>
</tr>
<tr>
<td>WAC5501</td>
<td>1989</td>
<td><em>Stemphylium sp.</em></td>
<td>narrow-leaved lupin</td>
<td>Badgingarra (WA)</td>
</tr>
<tr>
<td>WAC9984</td>
<td>1997</td>
<td><em>Stemphylium sp.</em></td>
<td>narrow-leaf lupin</td>
<td>Eyre Peninsula (SA)</td>
</tr>
<tr>
<td>WAC12986</td>
<td>2006</td>
<td><em>Stemphylium sp.</em></td>
<td>narrow-leaf lupin</td>
<td>South Perth (WA)</td>
</tr>
<tr>
<td>WAC13136</td>
<td>2007</td>
<td><em>S. botryosum</em></td>
<td>narrow-leaved lupin</td>
<td>South Perth (WA)</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Pathogen identification recorded in the Western Australian Culture Collection (WAC)

3.2.2 Isolation and purification of cultures

To culture and purify field isolates, leaflets were surface sterilized by dipping in 1% sodium hypochlorite for 1 minute and then rinsing in two series of sterile water before 5 × 5 mm pieces of tissue were excised from the leaflets, and placed on 2% water agar. Plates were incubated at 20±2 °C under a cool-white fluorescent light/dark cycle of 12 h each with periodic observation. To obtain single spore isolates, growing edges of the fungal hyphae developing from the leaflet pieces were transferred aseptically to Petri
dishes containing 20% V8 juice agar medium (200 mL V8 Juice, 5.4 g of CaCO$_3$, 20 g of agar and distilled water to a total volume of 1 L). The fungi were identified following sporulation and pure cultures were stored at 4 °C. Isolates from the Western Australian Culture collection were obtained as lyophilized vials. The cultures were revived on V8 agar plates and incubated under the conditions described above.

3.2.3 Virulence testing

Four *L. angustifolius* genotypes i.e. Danja (susceptible with low level segregating resistance), Mandelup (resistant), Unicrop (susceptible) and WALAN2333 (susceptible) (Thomas et al., 2011) were used in virulence testing. Seeds were sown in 140 mm diameter pots placed in a glass-house at 20±2 °C. Two week old seedlings were inoculated with conidial suspension of each of the isolates at a concentration of 2×10$^5$ spores/mL. Plants were sprayed to incipient runoff (5 mL per plant) with the conidial suspension using an airbrush sprayer (Paasche Airbrush Co. USA) operated with BOC 8000 air regulator (BOC Gas and Gear Australia) at 0.1 MPa. Control pots were sprayed with sterilized water. Following inoculation, pots were placed in a misting chamber for 24 hours, then placed in a glass-house maintained at 20±2 °C and observed over a two week period for the development of disease symptoms. Virulence was assessed on the basis of mean lesion sizes on 5 leaflets from each plant. Each *Stemphylium* isolate was classified into one of four groups: highly virulent (>5 mm), virulent (2-5 mm) moderately virulent (1-2 mm) and avirulent (no lesions). The experiment was repeated twice.

3.2.4 Morphological studies

V8 agar plates were inoculated with mycelial discs (5 mm diameter) taken from the growing edge of pure cultures. Cultures were grown under 12 h light/dark cycle at 20±2 °C. Conidia were taken from mature colonies, mounted using 4% erythromycin in 10% ammonium hydroxide stain, and examined for size and shape. Colour of conidia was recorded from a separate unstained set. The results presented are based on measurement of 50 mature conidia (±SD) at 200× magnification visualized using light microscope. The morphology of conidia was photographed with Olympus DP21® using Olympus cellSens® imaging software.
3.2.5 DNA extraction

Each culture was derived from a single spore of the original isolate and was subsequently maintained on V8 Agar plates. Cultures were incubated at 20±2 °C for 14 days. Mycelia were harvested by scraping the surface of the plates with a scalpel into 2 mL micro-centrifuge tubes.

Approximately 50 mg of mycelium for each sample was used for DNA extraction. Tissue homogenization was done in microcentrifuge tubes (2 mL) containing fungal mycelium and two stainless steel beads (5 mm), using Qiagen® TissueLyser-II™ bead mill (Qiagen Australia). DNA extraction of homogenized tissue was carried out using the Qiagen® DNeasy™ Plant-Mini kit according to the manufacturer’s instructions.

3.2.6 PCR amplification and sequencing

Internal Transcribed Spacer (ITS) primers 4 and 5 (White et al., 1990) were used to amplify sequences of the internal transcribed spacer region. ITS4 and ITS5 primers amplified the entire ITS rDNA region (5.8S) and parts of the 18S small and 28S large subunit rDNA. Reaction mixtures contained the following reagents: Sterile deionised water (25 µl), Bioline™ NH₄ Buffer (Bioline Australia) 1x (5 µl), MgCl₂ 2mM (2 µl), dNTPs, 1mM (5 µl), Primer ITS4 0.25µM (1.25 µl), Primer ITS5 0.25µM (1.25 µl), Bioline™ TAQ 0.050U/µl (0.50 µl), DNA 10ng/µl (10 µl).

PCR amplification of the ITS rDNA regions was conducted with reaction conditions described by Camara et al. (2002). PCR-amplified DNA fragments were fractionated using 1.5% agarose gels in 0.5× TAE buffer, and were viewed by ethidium bromide staining and UV illumination. The PCR products were purified with Qiagen Qiaquick™ PCR Purification Kit according to the manufacturers’ instructions. Purified DNA products were sent to the Australian Genome Research Facility (AGRF) for sequencing where reactions were performed using the Big Dye Terminator method. The sequences of both strands of each fragment were determined for sequence confirmation.

3.2.7 Sequence alignments and phylogenetic analyses

Linnaeus search tool in Geneious™ Pro 6.1 software (Biomatters Ltd, New Zealand) was used to perform BLAST search and the results were visualised using the Linnaeus viewer. In the Linnaeus viewer the results are displayed in the form of evolutionary
trees according to the National Center for Biotechnology Information (NCBI) taxonomy. Sequences of Genbank accessions labelled as top hits in the Linnaeus viewer were retrieved from the NCBI database. ITS sequences of the isolates were then compared with those of five species of Stemphylium genus and one species from Ulocladium genus obtained from Genbank. Sequence alignment was performed with the ClustalW alignment tool in Geneious™ Pro 6.1 software. The alignments were checked visually and improved manually where necessary. Maximum likelihood (ML) analyses of the ITS rDNA sequence dataset were performed with the PhyML 2.2 plugin for Geneious™ Pro 6.1 using the Tamura-Nei substitution model. The branches were supported by bootstrapping with 1000 replicates.

3.3 Results

3.3.1 Isolation and virulence of the isolates

Twenty seven samples from NLL and wild blue lupins exhibiting symptoms similar to GLS were collected from the northern wheat-belt of WA during the spring of 2009, 23 samples were collected from the same areas in 2010 and in 2011, 16 samples were collected from the Southern wheat belt of WA (Figure 3.1). No examples of severe infection were found during the survey, with only a small percentage of samples exhibiting classical GLS lesions. Only 14 of the samples collected during the period of three years were later shown to contain Stemphylium spp.

Seven of the 14 Stemphylium isolates collected were found to be avirulent (most probably saprophytes) whereas five others were moderately virulent and two isolates, collected from Eradu in 2011 (Eradu 11) and Medina 2010 (Medina 10) were highly virulent. Among the historical isolates, WAC12986 and WAC13136 were highly virulent, and WAC3719 was moderately virulent whereas the rest of the isolates were found to be avirulent (Table 3.2).
Figure 3.1: Sites in WA from where lupins were sampled for grey leaf spot disease over the period of three years (2009-2011)

In plants inoculated with virulent isolates, initial symptoms were observed on the fourth day after inoculation as ash coloured spots followed by development of grey necrotic spots within seven days. Regardless of whether the isolates were later identified as *S. botryosum*, *S. vesicarium*, *S. solani* or unidentified *Stemphylium* sp., they produced similar symptoms on leaflets of susceptible genotypes of NLL. The inoculated fungi were consistently isolated from the diseased plants, cultured, identified and re-inoculated to confirm the pathogenicity. None of the isolates caused disease on the resistant NLL variety Mandelup (data not presented).
3.3.2 Conidial morphology of the isolates

Conidial morphology recorded for the new and historical isolates separated the isolates into three distinct groups with the exception of two isolates, Geraldton 09 and Moonyoonooka 09. Group 1 had conidia with length to width (l/w) ratio of 1.5-1.7, oblong-ovoid shape and deep brown colour; group 2 with l/w ratio of 1.7-1.8, oblong-ovoid shape and medium brown colour; and group 3 with l/w ratio of 1.9-2.0, oblong-broadly ovoid shape and medium brown colour. Conidia of the outlier Geraldton 09 most closely resembled group 3, having l/w ratio of 1.9, and were oblong-broadly ovoid shaped but were deep brown in colour. The second outlier, Moonyoonooka 09 produced conidia with l/w ratio of 2.1 which were pale brown in colour and oblong in shape with a pointed apical cell, thus presenting a distinct morphology from the three groups. Table 3.3 provides the key morphological attributes of the relevant Stemphylium species. Group 1 showed the attributes of *S. botryosum*, group 3 matched the description of *S. vesicarium* and Moonyoonooka 09 was classified as *S. solani* (according to the attributes listed in Table 3.3). The two isolates from group 2 and the outlier Geraldton 09 did not match any of the above mentioned species (Table 3.2, Table 3.3).
Table 3.2: Conidial morphology, virulence and molecular characterisation of historical and new Western Australian isolates of Stemphylium from lupins

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Conidial Morphology</th>
<th>Genbank Match</th>
<th>Organism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dimensions (µm)</td>
<td>L/W ratio ± SD</td>
<td>Colour</td>
</tr>
<tr>
<td>Eradu 11</td>
<td>21-33 × 12-18</td>
<td>1.65 ± 0.12</td>
<td>Deep Brown</td>
</tr>
<tr>
<td>Mingenew 09</td>
<td>19-29 × 12-17</td>
<td>1.63 ± 0.14</td>
<td>Deep Brown</td>
</tr>
<tr>
<td>WAC12986</td>
<td>17-32 × 12-23</td>
<td>1.5 ± 0.24</td>
<td>Deep Brown</td>
</tr>
<tr>
<td>WAC13136</td>
<td>23-38 × 15-24</td>
<td>1.68 ± 0.10</td>
<td>Deep brown</td>
</tr>
<tr>
<td>WAC5462</td>
<td>18-28 × 11-17</td>
<td>1.64 ± 0.14</td>
<td>Deep Brown</td>
</tr>
<tr>
<td>Eradu 10</td>
<td>18-31 × 11-19</td>
<td>1.74 ± 0.19</td>
<td>Medium Brown</td>
</tr>
<tr>
<td>WAC5501</td>
<td>16-25 × 10-16</td>
<td>1.7 ± 0.24</td>
<td>Medium Brown</td>
</tr>
<tr>
<td>Medina 10</td>
<td>21-36 × 11-18</td>
<td>1.93 ± 0.25</td>
<td>Medium Brown</td>
</tr>
<tr>
<td>WAC3719</td>
<td>18-30 × 9-15</td>
<td>1.94 ± 0.22</td>
<td>Medium Brown</td>
</tr>
<tr>
<td>WAC9984</td>
<td>18-35 × 8-18</td>
<td>1.96 ± 0.32</td>
<td>Medium Brown</td>
</tr>
<tr>
<td>Walkaway 09</td>
<td>18-29 × 9-15</td>
<td>1.92 ± 0.21</td>
<td>Medium Brown</td>
</tr>
<tr>
<td>Geraldton 09</td>
<td>16-29 × 9-19</td>
<td>1.82 ± 0.27</td>
<td>Deep Brown</td>
</tr>
<tr>
<td>Mooyoonooka 09</td>
<td>24-39 × 11-18</td>
<td>2.1 ± 0.28</td>
<td>Pale Brown</td>
</tr>
</tbody>
</table>

*a* based on mean lesion sizes on leaflets on WALAN2333. “+++” highly virulent (>5 mm), “++” virulent (2-5 mm) “+” moderately virulent and “-” avirulent (no lesions)

*b* naming is based on corresponding Genbank match
Table 3.3: Published descriptions of *Stemphylium* species in the analysis (Simmons, 1969; Ellis and Gibson, 1975)

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Dimensions (µm)</th>
<th>L/W</th>
<th>Colour</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. botryosum</em></td>
<td>24-33 × 15-24</td>
<td>1.2-1.8</td>
<td>Deep brown</td>
<td>oblong-ovoid</td>
</tr>
<tr>
<td><em>S. vesicarium</em></td>
<td>25-42 × 12-22</td>
<td>1.5-2.7</td>
<td>Medium Brown</td>
<td>oblong-broadly oval</td>
</tr>
<tr>
<td><em>S. solani</em></td>
<td>35-55 × 18-28</td>
<td>2</td>
<td>Golden Brown</td>
<td>oblong-pointed apex</td>
</tr>
</tbody>
</table>

### 3.3.3 Molecular characterization of isolates

The ITS sequence sizes determined for the isolates ranged between 475-491 bp. When compared with the sequences in NCBI database through BLAST search, the sequences were found highly homologous to *Stemphylium* genus with the exception of one isolate WAC269, which was homologous to a closely related genus *Ulocladium*. The isolates closely matched with three species; *S. vesicarium; S. solani* and *S. botryosum (P. tarda)*; and two groups of unidentified *Stemphylium* sp. (Table 3.2). With the exception of one isolate of unidentified *Stemphylium* sp. (WAC12986), all other unidentified *Stemphylium* sp. isolates were identical, indicating that two separate unidentified *Stemphylium* sp. were present in this collection. WAC12986 was more closely related to *S. botryosum* than to other isolates unable to be classified to species. Similarly, isolates within either the *S. vesicarium* or *S. botryosum* groups were identical with all other group members in the sequence of the ITS region. One isolate (Mooyoonooka 09) was found to cluster with *S. solani*, confirming the morphological identification (Table 3.2). One isolate from the WAC collection (WAC269) which had been archived as *Stemphylium* sp. was found to be *Ulocladium* sp. from its sequence identification (Figure 3.2). Comparisons to sequences of the relevant species in the GenBank database revealed 100% nucleotide (nt) match to *Stemphylium* sp. (JX982415), *S. botryosum/P. tarda* (AF071345), and *S. solani* (AB693928) sequences, and 99% nt similarity to *S. vesicarium* (AM746021) (Table 3.2). The sequences of ITS1 region, 5.8S, and ITS2 region of rDNA of all the isolates, five species of *Stemphylium* and one species of *Ulocladium* were used to compile a data matrix for the maximum likelihood analysis. The phylogram produced by the analysis was rooted with the outgroup *Ulocladium* sp.
Figure 3.2: A phylogram representing the relationship of Western Australian lupin isolates to *S. botryosum*, *S. vesicarium* and *S. solani* based on ITS rDNA sequences (495bp) rooted with the outgroup *Ulocladium* sp. using maximum likelihood analysis performed with Geneious Pro 6.1 using PhyML plugin. Scale bar represents substitutions per site. Numbers adjacent to branches indicate the percent of support from 1000 bootstrap replications. Group designation is based on Table 3.2.

### 3.4 Discussion

The objective of this investigation was to survey the WA lupin growing regions for the occurrence of virulent *Stemphylium* sp. infecting lupins, identify and characterize the collected isolates, and historical isolates of *Stemphylium* using morphological and ITS rDNA sequences. The information that was available for the isolates from the WA Culture Collection indicated an association of *Stemphylium* with lupin crops in WA over nearly 50 years (Table 3.1). Records from Atlas of Living Australia (www.ala.org.au) show that most of the reports for *Stemphylium* are from WA and are from various hosts. This suggests the possibility of the endemic presence of *Stemphylium* in WA cropping areas and subsequent selection of isolates pathogenic to
NLL during the early domestication of the crop. However, the information available for the historic isolates was limited otherwise and species designation is assumed to be based on morphological studies at the time of collection.

Two historical isolates WAC12986 and WAC13136 were found to be highly virulent and one isolate WAC3719 was moderately virulent whereas the rest of the WAC isolates were avirulent. Ellis (1971) notes *S. botryosum* is a cosmopolitan fungus and is commonly isolated from air, paper, soil and dead herbaceous plants. Due to lack of evidence of virulence of the historical isolates, it can be suggested that most of the historical isolates tested were saprophytes.

Surveys undertaken during 2009-11 demonstrate the presence of the pathogen in the field. However, only seven out of 66 samples taken were found to be infected with virulent *Stemphylium* spp. and only a further seven samples revealed avirulent *Stemphylium* spp.. The majority of GLS symptoms therefore arose from alternative causes, which is a complication for field diagnosis.

Leaf spots that came from blue lupin (*L. consentini*) in the present study were found to be not caused by *Stemphylium* spp. infection. Preliminary alternate-host tests suggest that it is highly unlikely that blue lupin is host to any of the current regime of isolates/species of *Stemphylium* present in WA (data not presented). Blue lupins grow wild through many northern parts of the WA grainbelt, often adjacent to NLL crops, they are an important component of the epidemiology of another disease, lupin anthracnose, as they act as a reservoir of inoculum for this disease (Thomas, 2003).

Conditions for GLS have not been ideal in WA particularly because of a very low proportion of susceptible genotypes grown in the field (Thomas et al., 2008). Thus a low incidence of the disease was observed during the surveys. Virulence tests performed revealed only half of the 14 samples with associated *Stemphylium* had virulent *Stemphylium* isolates. Five of those were moderately virulent and only two were highly virulent. Of the two highly virulent isolates, one (Medina10) came out of the lupin breeding shadehouse and probably attained highly virulent status as a result of continuous build-up of inoculum over years of lupin planting in that location. Though the study demonstrates very low occurrence of virulent isolates of *Stemphylium* in the
field, it also suggests that inoculum build-up over time may result in appearance of highly virulent isolates of *Stemphylium*.

The NLL cultivar Mandelup exhibited resistance to the all virulent isolates consistent with its reported resistance. Similarly previously reported susceptible genotypes of *L. angustifolius* were found susceptible to all the virulent isolates (Thomas et al., 2011). This result indicates that re-emergence of the disease can be explained by lack of selection for resistance amongst some recent releases from the *L. angustifolius* breeding program rather than a genetic change in the pathogen enabling the existing resistance to be overcome.

Three different species of *Stemphylium* were identified using morphological and molecular techniques. One of the isolates collected during 2009 survey was found to be *S. solani* (Table 3.2; Figure 3.2) and the other two species causing disease in WA were identified as *S. botryosum* and *S. vesicarium*. Wells et al. (1962) suggested the association of *S. solani* with GLS disease of lupins in North America. Irwin et al. (1986) identified and morphologically characterized *Stemphylium* spp. collected from various hosts in Australia and reported differences in species causing the same disease in USA and Australia. Historically, in WA only *S. botryosum* and *S. vesicarium* were found associated with the disease (Forbes et al., 1975; Thomas et al., 2011) making this the first Australian report of the association of *S. solani* with the GLS disease. It is uncertain whether the discovery reflects a more thorough survey than in the past or an increased presence of *S. solani* in the WA environment.

Results from morphological studies were slightly different from molecular studies. Thomas et al. (2011) using morphological techniques identified WAC12986 as *S. botryosum* however, DNA analysis of the ITS rDNA did not support that designation, although it clustered with *S. botryosum* in Group 1 (Figure 3.2). Further analysis using additional DNA sequences should assist in the clarification of the identity of this highly virulent isolate. Further sequence analysis also will clarify the separate species status of these isolates, which is quite unusual given that they are all responsible for the same disease on NLL and are all rendered avirulent by the single gene resistance in cultivar Mandelup.
In conclusion, three species of *Stemphylium* i.e. *S. botryosum*, *S. vesicarium* and *S. solani* were associated with the GLS disease of lupins in WA and virulence of the isolates appeared independent of the species designation. All virulent isolates of *Stemphylium* caused similar disease symptoms regardless of the particular species they belonged to. The current study demonstrated the presence of diversity in populations of *Stemphylium* spp. infecting NLL. However, because the number of isolates of *Stemphylium* and genoytypes of *L. angustifolius* used in the virulence study were limited, a more detailed study on the diversity of *Stemphylium* spp. is warranted. Investigating the origin and distribution of *Stemphylium* and susceptibility of the current NLL cultivars in production will assist in devising strategies to reduce the primary inoculum of the pathogen and its subsequent spread to vulnerable populations of NLL and potentially to other lupin crops.
CHAPTER 4

Grey leaf spot of lupins: Temporal dynamics and yield loss in *Lupinus angustifolius* L.

Abstract

In lupins, *Stemphylium spp.* causes a disease known as grey leaf spot (GLS). In Western Australia (WA) the disease caused heavy yield losses during the 1970s but over the last four decades it has not been a significant constraint to lupin production due to the development and implementation of resistant cultivars. Recently, its occurrence has again been reported and a proportion of the existing breeding material no longer contains resistance to this disease. The current study was carried out to investigate the impact of the susceptibility of various genotypes of *Lupinus angustifolius* (narrow-leafed lupins) and different sources of inoculum, on the progress of the disease and grain yield loss in lupins. Four genotypes of narrow-leafed lupins (NLL); Danja, Mandelup, Unicrop and WALAN2333 were used in the study. Disease progress was rapid in susceptible genotype WALAN2333 and resulted in up to 64% yield loss. Two isolates of *Stemphylium*, WAC13136 and WAC12986, were used in various combinations with infested trash as inoculum. Disease progress was rapid in the plots that received trash treatment compared to spray only inoculum. Trash treatment combined with WAC13136 spray-inoculation was most detrimental to the crop and greatest yield loss was observed in this treatment. Infested trash and susceptible genotype were found as the most important factors in the progress of the disease and resultant loss of grain yield.

4.1 Introduction

Grey leaf spot (GLS) in lupins is caused by *Stemphylium spp.*, various species of *Stemphylium* e.g. *S. botryosum, S. vesicarium* and *S. solani* have been reported to cause the disease (Wells et al., 1956; Wells et al., 1961; Tate, 1970; Gladstones, 1977). The disease was damaging to Western Australian (WA) narrow-leafed lupin (NLL) production in the early 1970s which prompted the need for resistance breeding against the pathogen (Gladstones, 1977). The WA Department of Agriculture in collaboration with US Department of Agriculture, successfully incorporated resistance in local
cultivars (Gladstones, 1982). Over the last 40 years it has been inconspicuous in WA lupin cropping due to the introduction and continued breeding of resistant cultivars (Gladstones, 1994; Thomas et al., 2008). However, in 2007 the disease was again observed in experimental breeding plots of NLL in WA. A proportion of the existing breeding material and some recently released cultivars no longer contain resistance to this disease (Thomas et al., 2011). Given the historical significance of this disease in WA lupin production, the recent re-emergence of the disease, in parallel with the release of susceptible varieties could be damaging for lupin industry of WA.

The disease causes significant damage to the crop at a later growth stage in high rainfall regions (Gladstones, 1994; Cowling and Gladstones, 2000). Initial infection by the pathogen produces numerous light brown coloured circular lesions on leaflets, ranging in size from a pinpoint up to a millimetre in diameter (Wells et al., 1956) and advanced lesions grow up to 7 mm in diameter (Tate, 1970). Mature leaflets or seedling cotyledons exhibit circular or semi-circular greyish lesions with ash coloured centres. Immature lesions lack the lighter coloured centre. Pod or stem lesions initially appear circular, superficial and grey brown in colour surrounded by a grey halo but mature lesions are more penetrating, with a concave surface, no halo and up to 5 mm in diameter. Stem splitting may occur at a lesion under severe infection (Tate, 1970; Thomas et al., 2011).

Significant reductions in grain yield of lupins have been reported to occur from the GLS disease (Edwardson et al., 1961). However, there has been no precise estimate of the effect of variety or inoculum pressure on disease progress and its relationship with grain yield loss. Understanding the relationship of temporal disease dynamics with these factors is important. This information will provide a better understanding of the pathosystem and more efficient, sustainable and effective management strategies may be developed to reduce the impact of the disease on yield.

An understanding of yield loss in relation to development of plant disease allows better predictions of crop losses and development of improved disease management practices (Madden and Nutter, 1995). Disease–yield loss relationship can be determined by relating yield loss to disease intensity or disease progress curves (James, 1974). Data on the amount of disease on plants at different time intervals is collectively represented in the form of disease progress curves. Similar curves are produced by growth curve
models such as the logistic and Gompertz (van Maanen and Xu, 2003). In instances where a disease progression does not fit to the logistic model, fits are improved with the nonsymmetrical Gompertz model (Berger, 1981) or Richards models (Campbell and Madden, 1990).

Source, type and density of inoculum has a profound effect on the development of disease (van Maanen and Xu, 2003). Previous studies of GLS disease in lupins suggest involvement of trash in initiating the disease (Thomas et al., 2011). Trash-borne inoculum of *Stemphylium*, as occurs in lentils (Taylor et al., 2007) can result in multiple primary infection occurrences, causing an early and rapid development of disease. However, involvement of trash in the GLS disease of lupins has not been studied from this perspective.

Two experiments were conducted during this study. In one experiment, disease progress and yield loss were studied in a small but diverse group of NLL genotypes to determine the relationship between genotype resistance, disease progress and grain yield loss. The second experiment investigated the impact of inoculum source on disease progress and grain yield loss. Both experiments were conducted at two sites; at one site, the experiments were conducted under natural field conditions while at the other site, the experiments were conducted under shadehouse conditions.

### 4.2 Materials and methods

#### 4.2.1 Seed material

Four genotypes of NLL were used in the current study; 1. Danja (Susceptible with low level segregating resistant types-S-Seg), 2. Unicrop (Susceptible-S), 3. WALAN2333 (S) and 4. Mandelup (Resistant-R) (Thomas et al., 2011). The majority of Danja plants are susceptible, however a small proportion of individual plants are resistant. This genotype was included in the field study to represent a partially resistant response in a field population of plants. Seeds of these genotypes were obtained from the Department of Agriculture and Food Western Australia (DAFWA) Lupin Breeding Program, South Perth.
4.2.2 **Inoculum**

4.2.2.1 **Spray inoculum**

Two isolates of *S. botryosum*, WAC12986 and WAC13136 (Thomas et al., 2011), used in this study, were obtained from the Western Australian Culture Collection. Fresh cultures were prepared for each spray inoculation by growing the isolates on 20% V8 agar medium for 14 days at 22 ± 2 °C and 12 h light/dark cycle (Everts and Armentrout, 2001). For inoculum preparation spores were harvested in 1% Tween-20 solution, the number of spores per ml of solution was estimated by triplicate haemocytometer counts. Final concentration was adjusted to $1.50 \times 10^6$ spores/ml.

4.2.2.2 **Infested trash**

Infested trash consisting of lupin stems and leaves was produced during the 2010 cropping season. Plants of susceptible NLL genotype (WALAN2340) were grown in a shadehouse covered with fine insect proof mesh, resulting in higher relative humidity and temperature conditions. Plants were inoculated with conidial suspension of WAC13136. First inoculation was done one month after emergence and two subsequent inoculations were done at 28 days interval. Plants were sprayed to incipient runoff with the conidial suspension ($1.50 \times 10^6$ spores/ml) using an airbrush sprayer (Paasche Airbrush Co., Chicago, USA) operated with BOC 8000 air regulator (BOC Gas and Gear, Welshpool, Australia) at 0.1 MPa. At maturity, stems and leaves from the diseased plants were collected for use as trash for plant inoculation in the subsequent year.

4.2.3 **Experimental sites**

Experiments were conducted at two sites i.e. South Perth plots (SP) and Medina Research Station (MRS) of DAFWA during the winter season of 2011. These sites are approximately 25 km apart from each other. Experiments at SP were established under field conditions while at MRS experiments were established inside a shadehouse covered with translucent insect proof mesh (Figure 4.6 and Figure 4.7). At each site two experiments were conducted i.e. one investigating the effect of variety resistance on disease intensity and yield and the other investigating the effect of inoculum type (infested trash and/or spray-inoculation) on disease intensity and yield. Sowing occurred in the 2nd week of May 2011 at SP and in 1st week of June 2011 at MRS.
Daily maximum and minimum temperature and relative humidity were recorded for the MRS experiments by Tinytalk® data loggers (Gemini Data Loggers Ltd., West Sussex, UK) placed adjacent to the experimental plots inside the shadehouse. Daily maximum and minimum temperature and relative humidity for SP were downloaded from the DAFWA’s automatic weather station approximately 500m from the experimental site.

4.2.4 Experimental design

4.2.4.1 Variety resistance experiment

This experiment was designed to study the impact of disease on grain yield of different genotypes that have varying level of resistance against the disease. The trial was designed as a two factor split plot experiment. The two factors in the experiment were; 1. Infestation and 2. NLL genotype. Infestation had two levels; infested (trash added) and control (uninfested) treatment, whereas there were four NLL genotypes; Danja (S-Seg), Unicrop (S), WALAN2333 (S) and Mandelup (R). Infestation treatments were applied to main plots and the genotypes were assigned to subplots within the main plots. The two infestation treatments were randomly assigned to the main plots within each of the three blocks in randomized complete block design (RCBD). The subplot genotypes were then randomly assigned within each main plot thus randomization of subplot levels also occurred within each main plot.

A total of 24 hill-plots (30 cm diameter circular plots, with 15 seeds sown equidistantly around the circumference) were established within an area of 6.4 m by 3.9 m. Upon emergence the number of plants was adjusted to 10 plants per plot. Trash was spread evenly on infested treatment plots at a rate of 800 kg/ha two weeks after seedling emergence, whereas control plots did not receive trash. To minimize the interplot interference, the infested plots were separated from each other and control plots using buffer rows of a resistant NLL genotype –Tanjil.

4.2.4.2 Inoculum treatment experiment

This experiment was designed to study the impact of inoculum source on the GLS disease progress and grain yield loss in NLL. A total of eight treatments were investigated during this study. Treatments consisted of (1) infested trash, (2) infested trash and a single spray-inoculation with WAC13136, (3) infested trash and a single spray-inoculation with WAC12986, (4) two spray-inoculations with WAC13136, (5)
two spray-inoculations with WAC12986, (6) single spray-inoculation with WAC13136, (7) single spray-inoculation with WAC12986, and (8) uninoculated control.

A total of 24 hill-plots (30 cm diameter circular plots) were established in an area of 8.4 m × 3.9 m. Fifteen seeds of NLL genotype WALAN2333 (S) were sown equidistantly around the circumference of the plots. Upon emergence the number of plants was adjusted to 10 plants per plot. Treatments were randomized in an RCB with three replications for each treatment. Trash was applied two weeks after emergence to plots designated for treatments 1, 2 and 3. The first spray of the inoculum was applied six weeks after emergence and the second spray of inoculum was applied 10 weeks after emergence.

4.2.5 Disease assessment

Both disease severity and disease incidence were recorded for each experiment. The first assessment of disease severity and incidence was done 28 days after sowing. From then on assessment was done at 14 day intervals at the South Perth site whereas the interval between the assessments at Medina Research Station was 28 days. Assessment of disease severity was made randomly from five plants per plot. During first assessment five leaves were assessed from each plant. The second assessment was made on 10 randomly selected leaves per plant. Subsequent assessments were made from three levels of plant canopy i.e. upper, middle and lower each on a set of five random leaves. Level of disease severity was assessed on leaves according to a 0-5 rating scale for GLS disease of lupins (Thomas et al., 2011). Disease incidence was recorded as the percentage of diseased plants out of total plants per plot. Data of incidence and severity were used to calculate disease intensity (Cooke, 2006; Nutter Jr et al., 2006).

\[
\text{Disease Intensity} = \frac{\text{Disease Incidence (\%)} \times \text{Disease Severity Value (out of 5)}}{\text{Maximum disease Severity Value (i.e. 5)}}
\]

\[\text{DI} = (I \times DS)/5\]

Equation 4.1
4.2.6 Temporal analysis

Disease progress curves were developed for each treatment in each experiment. Area under disease progress curve (AUDPC) was calculated using Equation 4.2 (Shaner and Finney, 1977).

\[
\text{AUDPC} = \sum_{i=1}^{n-1} \frac{(DI_i + DI_{i+1})}{2} (t_{i+1} - t_i)
\]

Equation 4.2

Cumulative disease intensity values were used to plot the disease progress over time.

4.2.7 Regression analysis

Linear and non-linear regression models were used to study the progress of the disease over time. A simple linear regression model was used as the first step to explain the relationship between disease intensity and time.

For non-linear regression, models such as Logistic, Gompertz and Richards –commonly used in plant disease epidemiology studies –were employed to obtain the best fit for disease progress (Xu, 2006). General curve fitting procedure in NCSS\textsuperscript{TM} version 9.0 was used for the regression analysis (Hintze, 2013). Richards model used to improve the fits can be written as:

\[
DI = K[1 - B \exp(-rt)]^{1/(1-m)}
\]

Equation 4.3

where DI = Disease Intensity, K = asymptote of disease progress curve, B = Integration parameter, r = rate of disease increase, m = parameter for shape of curve and t = time of observation.

Coefficient of determination ($R^2$), adjusted coefficient of determination ($adjR^2$) and patterns of standardized residuals were used to compare the accuracy of the models and their relative appropriateness.

4.2.8 Yield assessment:

At maturity, pods were counted and hand harvested from each plot of both the experiments. Harvested grain weight for each plot was recorded and weight of a 100
seed sample was assessed for each plot. Actual grain yield in kg/ha was calculated for every plot by dividing total weight of harvested grain for that plot by the plot area. Actual yield loss was determined as the difference between the total grain weight per plot and the average grain weight in the corresponding control plot. The effect of NLL genotype and inoculum treatment on grain yield was determined by analysis of variance. Mean comparisons of grain yield among genotypes and among the treatments were performed according to Tukey’s highly significant difference (HSD) test at $P<0.05$ (Tukey, 1953). The relationship between yield-loss and disease intensity was determined by regression analysis of final disease intensity and yield loss for each treatment of the inoculum treatment experiments at each site. Linear regression analyses were performed by a least squares procedure. Coefficient of determination ($R^2$), mean square error and residual plot against predicted values were used to evaluate the accuracy and appropriateness of the fitted model.

4.3 Results

4.3.1 Disease progress

The disease progress (AUDPC) estimated as cumulative percentage disease intensity varied significantly ($P<0.05$) among different genotypes and among different inoculum treatments (Figure 4.1, Figure 4.2, Figure 4.3 and Figure 4.4).

4.3.1.1 Variety resistance experiment

No disease was observed in either infested or uninfested control plots of resistant genotype Mandelup at either site. For the remaining three genotypes a total of six disease progress curves were produced based on disease assessment data at both experimental sites. Disease progress was rapid and final disease intensity was significantly higher ($P<0.01$) in susceptible genotypes WALAN2333 and Unicrop compared to the segregating genotype Danja. The disease progress curves with best fit were described by Richard’s regression model. Values for disease parameters were also estimated using the Richards model and improved model fit curves were prepared. Out of six predicted disease curves produced by Richard’s model, five showed a positive skewness –value of shape parameter ($m$) was estimated between 0 and 2 –suggesting that increase in the absolute rate ($r$) was faster as the rate approached the inflection point and only Danja at MRS showed negative skewness ($m > 2$). The predicted curves
elaborated that at both sites, it was more than 85 days after sowing before the inflection point of the disease progress curves in Danja was reached whereas the inflection point of predicted disease progress curves in WALAN2333 and Unicrop were achieved by 75-80 days after sowing (Figure 4.1 and Figure 4.2).

Figure 4.1: The effect of different narrow-leaf lupin genotypes on disease progress of grey leaf spot of lupins over time at South Perth. Dots represent replicate disease intensity observations and lines represent predicted disease progress curves estimated by Richards regression model.
Figure 4.2: The effect of different narrow-leaf lupin genotypes on disease progress of grey leaf spot of lupins over time at Medina. Dots represent replicate disease intensity observations and lines represent predicted disease progress curves estimated by Richards regression model.

4.3.1.2 Inoculum treatment experiment

A total of 14 disease progress curves were analysed for this experiment. The disease progress curves with best fit were described by Richard’s regression model. Similar to the variety resistance experiments, the values for disease parameters were also estimated using the Richards model for this experiment and improved model fit curves were prepared. All model predicted curves for this experiment showed a positive skewness ($0 < m < 2$). The differences between treatments with and without trash were manifest in the rate of disease development and later in the season when final disease intensity was significantly higher ($P<0.01$) in plots where trash was used as the source of primary inoculum compared to spray only inoculum. Predicted curves showed that
disease progress curves for trash treatments reached the inflection point in 67-79 days compared to spray only inoculum treatment that required 82-89 days (Figure 4.3 and Figure 4.4).

Disease progress and final disease intensity were greater in plots that received inoculum containing WAC13136 compared to those that received WAC12986 inoculum. Two sprays of both WAC12986 and WAC13136 proved more effective in establishing the disease in the respective plots compared to a single spray-inoculation with each isolate.

No disease was recorded in the non-inoculated control plots, indicating that minimal inter-plot interference occurred between the plots during the disease measurement period.

![Image](image.png)

**Figure 4.3:** The effect of different inoculum treatments on disease progress of grey leaf spot in narrow-leaf lupin genotype WALAN2333 over time at South Perth. Dots represent replicate disease intensity observations and lines represent predicted disease progress curves estimated by Richards regression model. T1= Trash Only, T2= Trash+ WAC13136 Spray, T3= Trash+ WAC12986 Spray, T4= Two sprays of WAC13136, T5= Two sprays of WAC12986, T6= Single spray of WAC13136, T7= Single spray of WAC12986 and T8= Uninoculated Control.
Figure 4.4: The effect of different inoculum treatments on disease progress of grey leaf spot in narrow-leaf lupin genotype WALAN2333 over time at Medina. Dots represent replicate disease intensity observations and lines represent predicted disease progress curves estimated by Richards regression model. T1= Trash Only, T2= Trash+ WAC13136 Spray, T3= Trash+ WAC12986 Spray, T4= Two sprays of WAC13136, T5= Two sprays of WAC12986, T6= Single spray of WAC13136, T7= Single spray of WAC12986 and T8= Uninoculated Control

4.3.2 Yield response:

4.3.2.1 Variety resistance experiment

Grain yield and number of pods was significantly ($P<0.05$) affected by genotype resistance, infestation (infested and control treatment) and the interaction of the two factors. At both sites, grain yield and total number of pods were influenced by the NLL genotype whereas 100 grain weight was not significantly affected. Mandelup showed no significant reduction in number of pods and grain yield in infested plots compared to the
control plots. Among the three other genotypes, WALAN2333 showed greater reduction in number of pods and grain yield in infested plots than Danja and Unicrop. Yield losses ranged from 39% in Danja to 64% in WALAN2333 at South Perth, and 24% in Danja to 44% in WALAN2333 at Medina (Table 4.1). The significant interaction effect between genotype and disease infestation reflects the direct influence of resistance on yield response. Yield loss was non-significant in resistant Mandelup compared to the other lines which were partially or fully susceptible. At both sites, in the uninfested plots WALAN2333 was the highest yielding variety, however in the presence of infested trash, Mandelup was significantly higher yielding than the other varieties.

Table 4.1: The effect of grey leaf spot infestation on pod number, grain yield and 100 grain weight of different narrow-leaved lupin genotypes at South Perth and Medina

<table>
<thead>
<tr>
<th>Site</th>
<th>Genotype</th>
<th>Pods per plant</th>
<th>Yield (×10³ kg/ha)</th>
<th>100 grain weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Control</td>
<td>Infested A</td>
<td>Control</td>
</tr>
<tr>
<td>South Perth</td>
<td>Danja</td>
<td>25.30 b</td>
<td>16.37a bc</td>
<td>3.35 c</td>
</tr>
<tr>
<td></td>
<td>Mandelup</td>
<td>25.83 b</td>
<td>25.93 a</td>
<td>4.84 b</td>
</tr>
<tr>
<td></td>
<td>Unicrop</td>
<td>24.67 b</td>
<td>12.57a c</td>
<td>3.37 c</td>
</tr>
<tr>
<td></td>
<td>WALAN2333</td>
<td>45.33a</td>
<td>19.10a b</td>
<td>6.72a</td>
</tr>
<tr>
<td>Medina</td>
<td>Danja</td>
<td>21.24ab</td>
<td>18.43ab</td>
<td>2.55b</td>
</tr>
<tr>
<td></td>
<td>Mandelup</td>
<td>27.86a</td>
<td>24.76a</td>
<td>2.53b</td>
</tr>
<tr>
<td></td>
<td>Unicrop</td>
<td>18.67b</td>
<td>13.09a b</td>
<td>2.49b</td>
</tr>
<tr>
<td></td>
<td>WALAN2333</td>
<td>22.24ab</td>
<td>15.10a b</td>
<td>2.91a</td>
</tr>
</tbody>
</table>

A = for each site, treatments with different superscript letter are significantly different (P<0.05) for subplot factor (genotype) within the mainplot factor (infested or control treatment)
* represent significant difference (P<0.05) between the infested and control treatment.

4.3.2.2 Inoculum treatment experiment

There were significant differences in the number of pods and grain yield of various treatments but no significant difference was observed in the 100 grain weight. At both
sites, least number of pods and lowest grain yield were recorded from plots inoculated with infested trash plus WAC13136 spray-inoculation whereas single spray-inoculation with WAC12986 was least detrimental. Infested trash resulted in 35% and 49% yield loss at SP and MRS. Trash plus WAC13136 spray caused 47% yield loss at SP and 64% at MRS. Single spray inoculation with WAC12986 resulted in 6% yield loss at SP and 12% at MRS whereas its two sprays caused 22% and 29% yield loss at SP and MRS, respectively. WAC13136 single-spray caused 11% and 17% whereas two sprays caused 28% and 38% yield loss at SP and MRS, respectively (Table 4.2).

Table 4.2: The effect of different inoculum treatments on pod number, yield and 100 grain weight of narrow-leafed lupin genotype WALAN2333 at South Perth and Medina

<table>
<thead>
<tr>
<th>Treatment</th>
<th>South Perth</th>
<th></th>
<th>Medina</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pods per plant</td>
<td>Yield (×10³ kg/ha)</td>
<td>100 grain weight (g)</td>
<td>Pods per plant</td>
</tr>
<tr>
<td>Control</td>
<td>51.17ª</td>
<td>7.19ª</td>
<td>12.78</td>
<td>28.19ª</td>
</tr>
<tr>
<td>Trash</td>
<td>32.97ªf</td>
<td>4.67ªde</td>
<td>13.20</td>
<td>16.76ªde</td>
</tr>
<tr>
<td>Trash+ Spray of WAC12986</td>
<td>30.13ªf</td>
<td>4.33ªe</td>
<td>13.00</td>
<td>14.76ªe</td>
</tr>
<tr>
<td>Trash+ Spray of WAC13136</td>
<td>22.80ªg</td>
<td>3.78ªf</td>
<td>12.96</td>
<td>11.90ªf</td>
</tr>
<tr>
<td>Two sprays of WAC13136</td>
<td>34.57ªde</td>
<td>5.18ªcd</td>
<td>12.83</td>
<td>19.10ªd</td>
</tr>
<tr>
<td>Two Sprays of WAC12986</td>
<td>37.67ªcd</td>
<td>5.59ªc</td>
<td>13.35</td>
<td>22.38ªc</td>
</tr>
<tr>
<td>Spray of WAC13136</td>
<td>40.87ªc</td>
<td>6.41ªb</td>
<td>12.68</td>
<td>25.05ªbc</td>
</tr>
<tr>
<td>Spray of WAC12986</td>
<td>47.60ªb</td>
<td>6.73ªab</td>
<td>13.47</td>
<td>26.29ªab</td>
</tr>
</tbody>
</table>

ª = treatments with different superscript letter are significantly different \( P<0.05 \)

4.3.3  Yield –disease relationship

Relationship between the final disease intensity of inoculum treatment experiment plots and the grain yield loss in those plots (as percentage of control plots) was explained by a simple linear regression model. The regression coefficient was significant \( p< 0.05 \) for both experimental sites. Estimated yield loss ranged from less than 10% at
approximately 50% disease intensity to a maximum of approximately 60% yield loss at 100% disease intensity. A 50% yield loss was estimated at 90% disease intensity (Figure 4.5).

Figure 4.5: Effect of final disease intensity on grain yield loss in Stemphylium grey leaf spot infested narrow-leafed lupin genotype WALAN2333 at South Perth and Medina, explained by simple linear regression model

4.4 Discussion

The relationship between GLS disease development and grain yield loss in NLL has not been studied previously. This research was carried out to explore the influence of different sources of inoculum and susceptibility of the host genotype on the relationship between grain yield loss and GLS disease development in NLL.

GLS disease progress over time in NLL genotypes sown at both sites was explained by a sigmoid-shape model. Several epidemics caused by plant pathogens have been appropriately described by a sigmoid disease progress model (Bejarano-Alcazar et al., 1995; Navas-Cortes et al., 2000; Tan et al., 2007; Mukherjee et al., 2010). The Richards
model gave the best fit of the disease progress curves. All disease progress curves of GLS intensity for various treatments were analysed with the Richards model. Compared to logistic and Gompertz regression models, the Richards model consistently gave better fits of all the disease progress curves, making it ideal for studying GLS disease progress in lupins. The Richards model has been employed to describe disease progress in various plant diseases (Park and Lim, 1985; Lalancette et al., 1988; Carisse and Kushalappa, 1990; Navas-Cortes et al., 2000). It has more flexibility in fitting the disease progress curves compared to other non-linear regression models due to its shape parameter (Scherm and van Bruggen, 1993).

A number of factors involved in the appearance of foliar disease symptoms i.e. genotype susceptibility, primary and secondary inoculum, and their interactions were responsible for the observed differences between the disease progress curves. The disease progress varied depending upon the initial time it took to establish the infection. A slower initiation of infection resulted in delayed inflection point and lower final disease intensity rating. Disease progress curves for inoculum treatments involving trash appeared to be a distinct group of three curves with higher final disease intensity (Figure 4.3 and Figure 4.4) Disease progress was rapid in the trash treatments at the start and hence the final disease intensity was higher compared to spray inoculum. Early application of trash could have resulted in multiple primary infection events and a greater number of secondary infection cycles in the trash treatments compared to spray inoculum. Presence of infested trash at the beginning of the cropping season will provide an adequate quantity of inoculum to start establishment of infection at early stages of plant growth compared to any aerial inoculum, resulting in faster progress of the disease within the crop and higher final disease intensity. Differences among the curves produced by the two isolates were apparent suggesting that the virulence of the two isolates was slightly different. Morphological differences between the two isolates do occur (Thomas et al., 2011) and there could be difference in the physiology of the isolates. Cowling and Gilchrist (1982a) have reported differences in virulence of Stemphylium biotypes under different environmental conditions. However, there was no evidence that different environmental conditions had any impact on the difference in virulence of the isolates during this study as results at both the sites were consistent.
The intensity of the disease at the two sites was not directly compared due to the difference in time over which the progression was studied. However, difference in progression of the disease was apparent between sites from the disease progress curves (Figure 4.1, Figure 4.2, Figure 4.3 and Figure 4.4). Disease progression appeared to be faster at MRS compared to SP. The differences in the progress of the disease may be due in part to differences in weather conditions. Weather inside the shadehouse at MRS was slightly warmer (average daily temperature ranged between 10.2-32.4 °C) and humid (average daily RH ranged between 53-100 %) during the cropping season compared to SP (average daily temperature ranged between 6.3-26.7 °C and average daily RH range was 38.5-99.0%). Weather is anticipated to play an important role in the epidemic development (Thomas et al., 2011). For each site, differences in GLS disease development are related mainly to genotype susceptibility, source of inoculum and virulence of the pathogen isolate.

Reduction in grain yield by the disease was significant whereas reduction in 100-seed weight was not significant ($P>0.05$) (Table 4.1 and Table 4.2). Therefore, the overall grain yield loss caused by the disease is considered mainly as a result of significant decrease in the number of pods per plant and therefore a decrease in number of grains per plant rather than to a reduction in mean grain weight. This suggests that the disease directly reduced number of grains through infection and abortion of pods or developing flowers. Yield of NLL is highly variable and depends on number of pods per plant and grain weight (Dracup et al., 1998). Palta and Ludwig (1998) suggested that grain weight in NLL varies depending upon the number of pods per plant and plants with fewer pods tend to have higher grain weight. During pod development nutrients from other parts of NLL plant –especially from leaves –are transferred to the pods (Dracup, 2008) thus if there are fewer pods, each receive proportionately more nutrients and resultant grains weigh more. However, GLS also directly affects the foliage of the plant and mature lesions of the disease cause defoliation (Wells et al., 1961; Tate, 1970; Gladstones, 1977; Thomas et al., 2011). It is possible that due to defoliation, the plant has less nutrients to relocate to pods and seed weight does not increase despite lower pod numbers (Table 4.1 and Table 4.2).

The results show a significant relationship between NLL grain yield and the disease intensity at the end of the season (Figure 4.5). Grain yield decreased linearly with
increasing final disease intensity. A favourable environment promoting disease will increase the final disease severity, Gladstones (1977) observed that in WA yield losses were greatest in seasons with wet/warm springs which supported disease development. Higher disease intensity resulting in defoliation before and during the seed maturation has previously been reported to be primarily responsible for reduction in grain yield of lupins infected by *Stemphylium* (Edwardson et al., 1961).

Grain yield in infested sub-plots was determined by susceptibility of the NLL genotype and resultant intensity of the disease. The rate of grain yield loss may be attributed to NLL genotype or source and type of primary inoculum. The yield loss corresponded with the resistance status of the varieties, with yield of the resistant variety Mandelup being unaffected by presence of inoculum and yield of the segregating (Danja) and fully susceptible (Unicrop, WALAN2333) lines being significantly reduced in the presence of inoculum. Similar response has been reported against *Stemphylium* incited GLS disease in bitter lupins where grain yield was significantly higher in resistant varieties compared to varieties susceptible to the disease (Edwardson et al., 1961). Resistance was bred into WA varieties to limit disease development and limit yield losses, and this resistance has been successful over the past 30 years. Re-emergence of the disease is a consequence of not screening and selecting for GLS resistance, which has proven remarkably durable, and is not due to a mutation of the pathogen to overcome the resistance character (Thomas et al., 2011). This research has demonstrated that the introduction of susceptible varieties into the WA cropping system could be devastating in terms of grain yield loss to this disease and therefore the importance of maintaining resistance within the NLL breeding program gene pool.

The study incorporates the effect of NLL genotype susceptibility and virulence and source of primary inoculum, thereby accounting for the effects of different disease progress curve-associated variables on grain yield and providing a better understanding of the NLL–GLS interaction. The study suggests that under favourable conditions the disease can cause up to 60% loss in yield of NLL in WA. Presence of trash infested with a virulent isolate and susceptible genotypes in the cropping system are the most important contributing factors in the development and progress of the disease and, in their presence, only a favourable environment is needed to facilitate infection.
Figure 4.6: Grey leaf spot – yield loss assessment experiments (a) Field experiment at South Perth (b) Shadehouse experiment at Medina
Figure 4.7: Plants of narrow-leafed lupin genotype WALAN2333 showing symptoms of grey leaf spot disease (a) Field experiment at South Perth (b) Shadehouse experiment at Medina
CHAPTER 5

Influence of temperature and moisture on growth and sporulation of *Stemphylium*, and intensity of grey leaf spot disease in lupins

Abstract

This study was carried out to determine the influence of temperature and moisture on; (i) mycelial growth, sporulation and germination of conidia of *Stemphylium* *in vitro*, (ii) grey leaf spot (GLS) lesion development in a detached lupin leaf bioassay and (iii) GLS disease intensity validation in intact plants. Mycelial growth and sporulation of *Stemphylium* were assessed at five levels of Relative Humidity (RH) combined with five cyclic day/night temperatures. Sporulation was also assessed under five photoperiod conditions at five cyclic day/night temperatures. Optimum temperature and RH for mycelial growth were 27.5 °C and 95% respectively. Optimal conditions for sporulation of *S. botryosum* were diurnal light, moderate RH (85%) and temperature of 22.5 °C. Germination and germtube growth of conidia of *Stemphylium* were studied at five constant ambient temperatures. Optimum temperatures for conidial germination and germtube growth were 22.5 °C and 27.5 °C respectively. GLS lesion development on detached narrow-leafed lupin (NLL) leaves was assessed at five cyclic day/night temperatures maintained for eight leaf wetness durations. For GLS lesion development day/night temperatures of 25/20 °C were most favourable and lesions only developed at RH 90% or above. Finally, GLS disease intensity was assessed after four leaf wetness durations in glasshouse experiments using intact plants to validate the bioassay results. A minimum wetness period of 12 h is required to establish GLS infection in NLL *in vivo* at optimum temperature.

5.1 Introduction

Narrow-leafed lupin (NLL; *Lupinus angustifolius* L.) is a leguminous crop grown in many countries either as grain legume or fodder. The majority of the Australian production of NLL is from Western Australia (WA) where the sandy low nutrient soils across parts of the winter cropping zone preclude cultivation of other legume species but gain significant benefit from the inclusion of a leguminous component in the cropping rotation. Fungal pathogens have been one of the main constraints to
production of NLL. Potentially damaging fungal foliar diseases occurring in WA are anthracnose (*Colletotrichum lupini*) and brown spot (*Pleiochaeta setosa*) (Thomas et al., 2008). During early utilisation of NLL in WA cropping systems, a significant disease that concerned lupin growers was grey leaf spot (GLS) caused by *Stemphylium* spp. (Cowling and Gladstones, 2000). The pathogen causes lesions on leaves, stems and pods of infected plants and produces conidia as inoculum for secondary infections whereas primary inoculum comes from infected stubble. Initially the disease appears in basal region of infected plants and then spreads rapidly to healthy plants in the vicinity when suitable weather conditions prevail. Since the mid-1970s, the introduction of a major resistance gene into the NLL breeding program has meant that the majority of released cultivars have been resistant (Cowling and Gladstones, 2000), and this disease has not been a constraint to NLL production in WA in that time. Recently however there has been a re-emergence of disease in breeding plots that coincides with increasing proportion of susceptible lines within the NLL breeding program, prompting a new research effort (Thomas et al., 2011).

*Stemphylium* spp. have a wide host range; besides lupins they also cause various foliar diseases in crops including lentil, spinach, onion, tomato, alfalfa, asparagus, peppers, pear and clover (Smith, 1940; Rotem et al., 1966; Rao and Pavgi, 1975; Lacy, 1982; Irwin, 1984; Koike et al., 2001; Kim et al., 2004; Taylor et al., 2007; Kohl et al., 2009). The wide host range of this pathogen species suggests its adaptability to a wide range of conditions, however there is lack of research on the influence of abiotic environmental variables on the intensity of disease in lupins.

An understanding of the influence of environmental variables on the disease cycle is a major component in understanding the risk posed by a pathogen, informing the development of appropriate disease management strategies and development of disease forecast models. Temperature and moisture are the most critical environmental variables in the epidemiology of most fungal diseases. These variables act as a major driving force behind all the phases of the disease cycle. Presence of moisture on the leaf surface is a condition critical for the development of most foliar fungal pathogens (Huber and Gillespie, 1992). Temperature and moisture are primary environmental factors affecting mycelial growth, sporulation and conidial germination of *Stemphylium*, and development of disease on various hosts (Bashi and Rotem, 1974; Emery and English,
Air-dispersed conidia of *Stemphylium* germinate and then penetrate the leaf surface of many hosts. The presence of free water on the leaf surface at optimal temperatures promotes germination of *Stemphylium* conidia and results in rapid disease development (Bradley et al., 2003). Increasing duration of leaf wetness has been reported to increase severity of *Stemphylium* infection (Cowling and Gilchrist, 1982a; Montesinos et al., 1995; Suheri and Price, 2000). *Stemphylium* has been reported to sporulate abundantly under longer periods of leaf wetness (Bashi and Rotem, 1975b) resulting in high disease severity (Cowling and Gilchrist, 1982a; Lacy, 1982).

Cowling et al. (1981) reported that different biotypes of the pathogen causing the same disease in a host may have different optimal environmental conditions that are likely to be influenced by the environment of the area of origin of the pathogenic biotype. Several studies have reported the influence of environmental variables such as photoperiod, rainfall, temperature and relative humidity (RH) on the *in vitro* growth and sporulation of *S. botryosum* and disease severity in various hosts. However, specific temperature and moisture conditions necessary for growth of the pathogen and spore germination in lupins have not been reported. Sporulation and conidial germination are crucial for the development of primary infection and an understanding of environmental effects on these critical processes is essential to ecological studies of plant–pathogen interactions. This information is necessary to develop a forecasting system to implement an integrated approach to disease control.

The objectives of this study were: to determine more precisely the influence of temperature and photoperiod on sporulation and influence of temperature and relative humidity on growth and sporulation of the pathogen; to determine the influence of temperature and incubation period on conidial germination and germ tube length; to determine the influence of temperature and wetness period on infection and development of GLS.

### 5.2 Materials and methods

#### 5.2.1 General

The study was divided into five parts. The first three parts involved assessing the effects of combination of photoperiod, temperature and RH on hyphal growth, sporulation,
germination and germtube elongation of *Stemphylium in vitro*. In the first, sporulation was assessed at five cyclic day/night temperatures under five photoperiods. In the second part, mycelial growth and sporulation were assessed at five RH combinations with five cyclic day/night temperatures. In the third part, germination and germtube growth of conidia of *Stemphylium* were assessed at five constant ambient temperatures. The last two parts looked at development of GLS disease. In the fourth part, GLS lesion development on detached NLL leaves was assessed at five cyclic day/night temperatures under eight durations of leaf wetness period. Finally, the optimum conditions of leaf wetness duration were validated in glasshouse experiments using intact plants.

### 5.2.2 Isolates and inoculum production

Two isolates of *S. botryosum* from the Western Australian Culture Collection (WAC12986 and WAC13136) were used in the experiments. *In vitro* mycelial growth, sporulation, germination and germ tube growth studies were performed with WAC13136 whereas inoculations of excised leaves and intact plants were carried out with both isolates separately. The isolates were originally isolated from NLL and identified as *S. botryosum* by Thomas et al. (2011). Subsequent molecular identification techniques have supported identification of WAC13136 as *S. botryosum* but WAC12986 was not classified as *S. botryosum* but did cluster with this group in genetic analysis (Chapter 3).

Conidia of *Stemphylium* were produced on petri dishes containing 20% V8 juice agar medium (200 mL V8 juice, 5.4 g of CaCO₃, 20 g of agar and sterile deionised (DI) water to a total volume of 1 L). For inoculation conidial suspensions were prepared from 7 to 10 day old cultures by adding sterile DI water, rubbing the culture surface gently with a bent glass rod, and filtering through Whatman Filter paper No. 4. The suspensions were adjusted with sterile DI water to desired concentrations determined using a haemocytometer. Tween 20 (one drop per 100 ml) was added to the inoculum suspensions as a wetting agent.

### 5.2.3 Plant material

Two NLL varieties; Mandelup (Resistant) and WALAN2333 (Susceptible) (Thomas et al., 2011), were used in all inoculation experiments.
5.2.4 Temperature and photoperiod regulation

Five economy incubators (Linder and May Co., Brisbane, Australia) were programmed at 12 h cyclic temperatures of 15/10, 20/15, 25/20, 30/25, and 35/30 °C with a 24 h photoperiod. For the light source, near ultraviolet (NUV) radiation was provided by Philips TL 40 W/08 black light tubes. Two 40 W fluorescent black light tubes were placed in a vertical position 20 cm apart. Five photoperiod conditions of 0, 10, 12, 14 and 24 h light per day, were maintained. The treatment of 0 h light was achieved by wrapping the Petri dishes in aluminium foil. Petri dishes for the alternating light conditions (10, 12 and 14 h) were wrapped and unwrapped in aluminium foil every day according to the treatment.

5.2.5 Relative humidity (RH) regulation

Plastic storage boxes with transparent clip-on plastic lid and opaque base of 36 cm x 32 cm x 14 cm were used as the RH control units. Highly saturated solutions of glucose, NaNO₂, NaCl, KCl and KNO₃ were used to maintain humidity in the boxes to 55±3, 65±3, 75±3, 85±3 and 95±3% respectively as described by Winston and Bates (1960). The saturated solutions were poured in the boxes to 4 cm depth. Aluminium wire mesh adjusted to the size of the boxes was placed above the salt solution in each box to hold the experimental material. The boxes were made air tight to maintain interior humidity by applying paraffin wax between the lid and base of the boxes. RH in each box was recorded with a Tinytalk RH data logger (Gemini Data Loggers Ltd., West Sussex, UK). RH control boxes were placed inside economy incubators (Lindner and May Co., Brisbane Australia) set at the required temperatures.

5.2.6 Physiological studies of the fungus

5.2.6.1 Effect of temperature and photoperiod on sporulation of Stemphylium

To determine the influence of temperature and light on the sporulation of Stemphylium, five cyclic temperatures 15/10, 20/15, 25/20, 30/25 or 35/30 °C and five photoperiod conditions of 0, 10, 12, 14 or 24 h light per day, were investigated. Conditions were maintained during the experiment as described above. After 10 days of incubation, spores produced in each Petri dish were washed with DI water into a glass beaker. Volume of the spore suspension was adjusted to 25 ml and concentration of the spores
per ml was estimated using a hemocytometer. The concentration thus obtained was used to calculate the total number of spores for each Petri plate.

5.2.6.2 Effect of temperature and relative humidity on mycelial growth and sporulation of Stemphylium

Mycelial growth and sporulation by the fungus were studied at five different temperature regimes; 15/10, 20/15, 25/20, 30/25 and 35/30 °C day/night temperatures, and five levels of RH; 55, 65, 75, 85 and 95%, as described above. Radial mycelial growth of the fungus in 90 mm diameter Petri dishes was recorded 10 days after inoculation of the Petri plates with 5 mm plugs of the fungal culture. Radial mycelial growth was measured along two axes at right angles to each other. After measuring the mycelial diameter, spore production in each Petri dish was estimated using a hemocytometer as described above, to estimate spore production per mm² of mycelium.

5.2.6.3 Effect of temperature on conidial germination and length of germ tube

Conidial suspension of $1 \times 10^4$ conidia per ml was prepared from seven days old cultures of both the isolates grown at 22±3 °C on V8 agar medium. Five ml of the suspension was poured into each Petri plate containing a thin film of water agar. The plates were made airtight with Parafilm (Grale Scientific Australia) to prevent drying. Plates were incubated at 12.5, 17.5, 22.5, 27.5 and 32.5 °C. Treatments were arranged in a completely randomised design. Conidial germination was recorded at 2 hour intervals with the aid of a compound microscope at 200x magnification. The germination percentage was determined by evaluating 200 conidia. Conidia were considered to have germinated when at least one germ tube of at least 5 µm length was present. The majority of spores had 2-3 germ tubes. Germ tube lengths of 50 conidia were recorded. In the case of more than one germ tube, the length of the longest germ tube was considered. The experiment was repeated twice and data were pooled for analysis.

5.2.7 Effect of temperature and wetness period on disease development in excised leaves

5.2.7.1 Inoculation of excised leaves

Plants of two NLL genotypes, Mandelup and WALAN2333, were grown from seed in 10 cm diameter plastic pots containing 2 L of potting mixture. Fully expanded leaves
were excised from the two week old plants. Leaves were excised close to the stem to retain maximum length of the petiole. Sarstedt 5 ml tubes (Cat. No. 60.558) were used to hold the excised leaves. Tubes were filled with 4.5 ml DI water. The opening of the tube was covered with Parafilm™ (Cat. No. PM996 Grale Scientific Australia). A hole was punched in the middle of the Parafilm™ covering to insert the petiole, ensuring that more than 1 cm of petiole was immersed in DI water (Figure 5.8).

Inoculum suspension was prepared from 10 day old cultures of both *Stemphylium* isolates as described above. Inoculations were performed using 10 µl transferpettor (Brand GMBH + Co, Germany). Two µl of $5 \times 10^4$ spores/ml suspension was dispensed on a flat clean surface portion of the leaflet. Inoculations with the same spore suspension were also performed on intact leaves of both varieties in the glasshouse, the inoculated leaves were then excised from the seedlings and included in the experiment. Two µl of DI water were dispensed on the leaves of both varieties to serve as a negative control.

### 5.2.7.2 Temperature and wetness period regulation

Inoculated leaves were placed in boxes similar to humidity control boxes but instead of salt solutions the boxes contained DI water and lids of the boxes were sprayed with fine mist to maintain free moisture in the microenvironment of the boxes. During the preliminary studies of symptom development at various RH values, it was observed that lesions were formed only when RH exceeded 90%. These boxes were placed in incubators set at 15/10, 20/15, 25/20, 30/25 and 35/30 °C day/night temperature under a 12 h photoperiod. Inoculated leaves were subjected to wetness periods of 6, 12, 18, 24, 30, 36, 42 and 48 h. After the wet treatment leaves were transferred to 75% RH box for development of symptoms at room temperature of about 20±2 °C. Symptom severity was recorded on each leaf 72 h after inoculation.

### 5.2.8 Effect of wetness period on disease development in intact plants

Seeds of two NLL genotypes 1) Mandelup and 2) WALAN2333 were planted into 10 cm diameter pots (four plants per pot) containing 2 L of potting mixture. Plants were grown under natural light in the glasshouse at ambient temperature averaging 20-25 °C. Inoculations were performed with two week old NLL seedlings that had four leaves.
Plants were sprayed to incipient runoff with the conidial suspension using an airbrush sprayer (Paasche Airbrush Co., Chicago, IL, USA) operated with BOC 8000 air regulator (BOC Gas and Gear, Welshpool, WA, Australia) at 0.1 MPa.

Plants were incubated during the wetting period in moist chambers. Moist chambers consisted of closed transparent plastic cages placed in metal trays filled with water to provide 100% RH. Plastic cages were fitted with Defensor 505 portable humidifiers (JS Humidifiers plc, West Sussex, UK) to maintain wet environment. After the wetness period, moistened plants were allowed to air dry at room temperature (about 20±2 °C), and then transferred to the glasshouse at 18-26 °C. Uninoculated controls were similarly handled, except they were sprayed with sterile distilled water plus 1% Tween 20. Pots were exposed to 6, 12, 24, 36, and 48 h wetness.

5.2.9 Data analyses

5.2.9.1 Physiological studies of the fungus

5.2.9.1.1 Effect of temperature and photoperiod on sporulation

Spores produced by the fungus were recorded as conidia per Petri plate. The experiment was repeated twice and after preliminary analysis of variance (ANOVA), data were pooled for analysis.

5.2.9.1.2 Effect of temperature and relative humidity on mycelial growth and sporulation

Radial mycelial diameter was recorded in mm after 10 days along two axes at right angles to each other. Sporulation was recorded as conidia per mm$^2$ of mycelium. The experiment was repeated twice and after preliminary ANOVA data were pooled for analysis.

5.2.9.2 Statistical procedure

Bartlett’s test was performed to check homogeneity of variances with CoStat 6.4 (CoHort Software, Monterey, CA, USA). ANOVA was performed using two factor (either temperature and light, or temperature and RH) completely randomised design. Analysis was done on untransformed data. Means were compared using Fisher’s least significant differences (LSD) test. Genstat 15 (VSN International Ltd, Hemel Hempstead, UK) was used for ANOVA and LSD test.
5.2.9.2.1 Effect of temperature on conidial germination and length of germ tube

Conidial germination was recorded as percentage and germ tube growth was recorded in µm. The experiment was repeated twice and similarity among experimental repeats was tested by ANOVA considering experimental repeats as blocks. Data from repeated experiments were pooled due to non-significant variance among the repeats. Linear and non-linear regression analyses were performed to explain the change in germination percentage and length of germ tube separately after the different incubation periods at various temperatures. Based on significance of estimated parameters \( p < 0.05 \), coefficient of determination \( (R^2) \), adjusted coefficient of determination \( (adjR^2) \), Shapiro-Wilk test, Wald test and patterns of residuals, the Gompertz regression model (Equation 5.1) suggested by Winsor (1932) was employed to describe the relationship (Lapp and Skoropad, 1976), using NCSS 9 (NCSS LLC, Kaysville, Utah, USA).

\[
y = ke^{-a-bt}
\]

Equation 5.1

where \( y \) is the germination percentage of conidia, \( k \) is upper asymptote or predicted maximum germination percentage, \( t \) is the time, \( a \) and \( b \) are constants that determine the rate of germination. The point of inflection \( (t_i) \) is where the rate of germination was maximum. Point of inflection \( (t_i) \) was estimated by Equation 5.2.

\[
t_i = \ln(a) / b
\]

Equation 5.2

5.2.9.2.2 Effect of temperature and wetness period on disease development in excised leaves

Severity of the disease was recorded on the basis of lesion diameter measurement. Data for lesion diameter was treated as relative lesion size of the maximum lesion size recorded (Pima, 1987). Percentage values of relative lesion size were used for the data analysis. The experiment was repeated twice and similarity among experimental repeats was tested by ANOVA considering experimental repeats as blocks. Data from repeated experiments for both isolates were pooled due to non-significant variance among the repeats and between the isolates. Regression analyses were performed to explain the influence of wetness duration on relative lesion size at various temperatures. Based on
significance of estimated parameters ($p < 0.05$), coefficient of determination ($R^2$), adjusted coefficient of determination ($adjR^2$), Shapiro-Wilk test, Wald test and patterns of residuals, the Gompertz regression model (Equation 5.1) was employed to describe the relationship between disease severity (relative lesion size) and duration of wetness period at different cyclic temperatures. The inflection point i.e. time when rate of increase of lesion size was maximum, was calculated using Equation 5.2.

5.2.9.2.3 Effect of wetness period on disease development in intact plants

Disease severity was recorded as percentage of the foliage showing disease symptoms (Nutter Jr et al., 2006). Data were recorded at two day intervals until two weeks after inoculation. Each treatment was applied to three pots with four seedlings each; this experiment was conducted three times. Because ANOVA did not show significant differences between experiments, regression analysis was performed on the pooled data of both isolates from the three experiments.

The Gompertz regression model (Equation 5.1) was employed to describe the progress of the disease over time after various wetness treatments. The inflection point i.e. time when rate of disease progress was maximum was calculated using Equation 5.2.

5.3 Results

5.3.1 Effect of temperature and photoperiod on sporulation

Sporulation was significantly ($P<0.05$) affected by temperatures and photoperiod and their interaction as shown in Figure 5.1. Maximum sporulation was observed at 25/20 °C under diurnal light settings. There was minimal sporulation at 35/30 °C, although this temperature was optimum for sporulation under 0 h light. A 5 °C increase in temperature from optimum reduced the sporulation by about 50% at 30/25 °C and more than 95% at 35/30 °C. Reductions of 5 °C from optimum reduced sporulation by 40% (20/15 °C) and by about 70% (15/10 °C). Significantly lower sporulation was observed at 0 h and 24 h light compared to the three other photoperiod conditions. There was no significant difference in sporulation under 10 h, 12 h or 14 h light per day.
Figure 5.1: The effect of different temperature and photoperiod regimes on sporulation of *S. botryosum*. Error bars represent standard error of means

5.3.2 Effect of temperature and relative humidity on mycelial growth and sporulation

Radial mycelial growth (Figure 5.2) and sporulation (Figure 5.3) were significantly (*P*<0.05) affected by differences in incubation temperature, RH and the interaction of these two variables. Mycelial growth was observed at all temperature and RH combinations. Within the parameters tested in these experiments, changing RH had a more significant impact on mycelial growth than changing temperature. Maximum mycelial growth occurred at 30/25 °C at all RH except 55% RH where maximum was at 25/20 °C. At all temperatures, increasing RH from 55 to 95% resulted in faster mycelial growth. Growth at 55% RH was less than 50% of maximum growth at all temperatures. While both RH and temperature impacted on sporulation, in contrast to mycelial growth, temperature appeared to be the major determining factor. At all RH, maximum sporulation was recorded at the 25/20 °C temperature regime, nearly double the amount at other temperature regimes. Increasing temperature above 30 °C significantly reduced sporulation, at 35/30 °C minimal sporulation was measured at all RH except 95% RH where there was no sporulation and only whitish mycelial growth was observed. At all
temperatures except 35/30 °C, a similar trend was evident, with maximum sporulation occurring at 85% RH and minimum sporulation at 55% RH. The greatest difference in sporulation between RH regimes was evident at optimum temperature of 25/20 °C.

![Graph showing the effect of temperature and relative humidity on mycelial growth of S. botryosum. Error bars represent standard error of means.](image)

**Figure 5.2:** The effect of temperature and relative humidity on *in-vitro* mycelial growth of *S. botryosum*. Error bars represent standard error of means.
5.3.3 Effect of temperature on conidial germination and germ tube elongation

Conidial germination was significantly ($P<0.05$) affected by temperature, incubation time and the interaction of the two variables as shown in Figure 5.4. Prediction limit of 95% using Gompertz regression equation revealed that rate of conidial germination varied significantly between incubation temperatures. Maximum rate of germination was recorded at 22.5 °C whereas slowest germination rates were at 12.5 °C and 32.5 °C. Difference between curves for 12.5 °C and 32.5 °C was non-significant at the 95% prediction limit. Similarly there was non-significant difference between curves for 17.5 and 27.5 °C at the 95% prediction limit. Time to 50% germination ranged from 9 hours at 22.5 °C to more than 12 hours at 32.5 and 12.5 °C.

Length of the germtube was significantly ($P<0.05$) affected by temperature, incubation time and the interaction of the two variables as shown in Figure 5.5. Length of the germtube increased with increasing temperature to the optimum temperature of 27.5 °C.
Gompertz regression curve for 27.5 °C was significantly different from all other temperatures at the 95% prediction limit. Difference between curves for 22.5 and 32.5 °C was non-significant at the 95% prediction limit. While the increase in temperature to 32.5 °C resulted in significantly smaller germ tube length than at 27.5 °C, this was still close to the optimum, as opposed to conidial germination where this temperature was proportionally less favourable.

Figure 5.4: Impact of temperature and incubation time on germination of conidia of *S. botryosum*. Predicted curves were estimated by the Gompertz regression model for different temperatures. Shading indicates the 95% prediction limit for the respective curve.
Figure 5.5: Impact of temperature on the rate of increase in length of the germ tube emerging from conidia of S. botryosum. Predicted curves were estimated by the Gompertz regression model for different temperatures. Shading indicates the 95% prediction limit for the respective curve.

5.3.4 Effect of temperature and wetness period on disease development in excised leaves

Development and size of lesions were significantly affected by temperature, wetness duration, and their interaction. There were no lesions observed on leaves of Mandelup (resistant) whereas leaves of WALAN2333 (susceptible) developed lesions of various
sizes at different combinations of temperature and wetness duration. Relative lesion size was calculated as percent size of maximum lesion size (about 5 mm). Relative lesion size was greater under increased wetness duration at all temperature combinations (Figure 5.9, Figure 5.10 and Figure 5.11). Increase in relative lesion size with increasing wetness duration was described by the Gompertz regression model. During the preliminary studies of lesion development at different RH values, it was observed that lesions were formed only at 90% RH or above (data not presented). Relative lesion size increased with increasing temperatures to a maximum at 25/20 °C and decreased above 30 °C. Lesion development was delayed with shorter wetting periods and at lower or higher temperatures than 25 °C. Maximum lesion size was achieved after 42 h wetness at all temperatures. Maximum lesion size was recorded for 25/20 °C after 42 h wetness followed by 30/25 °C whereas the smallest lesion size was recorded for 15/10 °C. Maximum rate of increase in lesion size as estimated by the Gompertz model was reached after 17 h at 25/20 °C whereas for 15/10 °C the maximum rate was achieved after 25 h. Lesions did not develop in the absence of a period of leaf wetness (Figure 5.9), after 6 h moisture, infection was evident, however relative lesion size was approximately 10% of maximum (Figure 5.10). There was no lesion evident on leaves inoculated with DI water only and no difference in lesion development between inoculation of leaflets pre or post excision.
Figure 5.6: Impact of temperature and wetness period on relative lesion size on detached leaves incubated with conidial suspensions of *S. botryosum*. Predicted curves were estimated by the Gompertz regression model for different temperatures. Shading indicates the 95% prediction limit for the respective curve.

5.3.5 Effect of wetness period on disease development in glasshouse grown intact plants

There was a significant effect of wetness duration on severity of the disease on intact plants (Figure 5.12). No symptoms were observed on any plant of the resistant variety Mandelup under any wetness treatment. For WALAN2333, disease severity increased with increasing wetness duration. No symptoms were evident in plants with only 6 h wetness exposure. Whitish grey spots started appearing on the fourth day after inoculation on leaves of plants subjected to the 12 h wetness period. Similar symptoms
were visible after two days in plants receiving 36 and 48 h wetness period. Four days after inoculation irregular light brown lesions on petioles and stems appeared in these plants. Petioles at the basal stem nodes started breaking 48 h after the appearance of characteristic symptoms, causing defoliation at the base of the seedling. Maximum disease severity was recorded 12 days after inoculation for the 48 h wetness treatment. Maximum rate of disease progress as estimated by the Gompertz model was reached four days after inoculation in plants that received the 48 h wetness treatment whereas for 12 h wetness treatment the maximum disease progress rate was attained six days after inoculation. Gompertz model fit curves for 36 and 48 h were not significantly different from each other but were significantly different from 12 and 24 h at 95% prediction limit (Figure 5.7). Disease severity increased with time up to 14 days after inoculation at the wetness periods tested. Maximum disease was evident by about 10 days after inoculation. Disease severity in plants receiving 12 and 24 h leaf wetness reached 50% after 8 days whereas plants exposed to 36 and 48 h wetness achieved 90% disease severity after the same period. Plants not severely infected continued to grow actively in the glasshouse, and the rate of disease progress slowed down with an increase in healthy tissue.
Figure 5.7: Effect of wetness duration on the development and severity of GLS on intact plants spray inoculated with *S. botryosum* spore suspension under glasshouse conditions. Predicted curves were estimated by the Gompertz regression model for different temperatures. Shading indicates the 95% prediction limit for the respective curve.

5.4 Discussion

Temperature and photoperiod were shown to play an important role in sporulation of *Stemphylium* grown *in vitro* (Figure 5.1). Diurnal photoperiod pattern is vital for initiation of sporulation and temperature also plays its supportive role in either effectively increasing sporulation or its inhibition under diurnal settings. While sporulation occurred at lower temperatures of 15/10 °C under continuous light (24 h), sporulation was inhibited or suppressed at higher temperatures. These results differ from those of Bashi and Rotem (1975a) who reported maximum sporulation in *S.*
botryosum on tomato leaves under 24 h light. However, the results are in agreement with Leach (1968) who reported inhibition of conidia formation under continuous light and higher temperature. It was suggested by Leach (1968) that there are two distinct phases of spore formation in S. botryosum. The inductive phase that results in production of conidiophores is stimulated by light whereas the terminal phase, which initiates the formation of conidia, is inhibited by light. Hence, the results indicate that diurnal settings that support both phases are necessary for the production of conidia. Higher temperatures also inhibit the formation of conidia by suppressing the terminal phase (Leach, 1967). This was evident from the results (Figure 5.1) as even under the diurnal settings at temperatures more than 30 °C, sporulation was significantly reduced.

Temperature and relative humidity in combination also are important environmental factors influencing mycelial growth and sporulation in Stemphylium. Figure 5.2 indicates that high day/night temperatures of 30/25 °C and high RH (95%) are optimum for mycelial growth whereas slightly lower day/night temperatures of 25/20 and 85% RH support sporulation in the fungus (Figure 5.2). These results are in accordance with Leach (1967) who reported suppression of sporulation at temperatures above 30 °C and Hosen et al. (2009), who reported 25 °C as the optimum temperature for mycelial growth of S. botryosum. Sterile conidiophores were produced at high temperatures (Figure 5.3). Similarly, Kim et al. (2004) reported absence of sporulation in S. solani at 30 °C.

Conidial germination and length of the germtube were significantly affected by the incubation temperature (Figure 5.4 and Figure 5.5). Optimum temperature for conidial germination was found to be 22.5 °C (Figure 5.4). Increase in temperature above this resulted in a decrease in germination percentage. These results are in agreement with those reported by Sivan and Barkai-Golan (1976) for S. botryosum. Mwakutuya and Banniza (2010) reported an increase in germination percentage of conidia with the increase in temperature up to 30 °C whereas Hosen et al. (2009) suggested that the conidial germination percentage for S. botryosum varies from region to region within a wide range of temperature from 20 to 30 °C. Optimum temperature for elongation of germtubes in this study was 27.5 °C (Figure 5.5). This difference in temperature for germination of conidia and the elongation of germtubes suggests diurnal behaviour of the pathogen. Lower temperatures at night time may favour the germination of the
conidia but higher day temperatures are required for the elongation of the germ tube and penetration of the host. At optimum temperature of 22.5 °C, 50% germination of conidia was achieved after 9 hours of incubation. Jakhar et al. (1996) observed that the conidia of *S. vesicarium* required a lag period of at least 2 h for germination and about 5% conidia germinated in 4 h. However, in our study about 20% conidia germinated at 22.5 °C.

Temperature and moisture are observed as significant environmental factors influencing infection and lesion size in a bioassay using excised NLL leaves (Figure 5.6). There was no lesion formation at RH below 90% (data not presented). The results indicate that the effect of the length of the wetness period resulting in light or severe infection depends on the temperature, with an optimum at 25/20 °C. At temperatures lower or higher, longer periods of wetness were required for significant infection. *Puccinia melanocephala* infection in sugarcane followed a similar trend. Disease severity was low at 15 and 31 °C regardless of leaf wetness duration. No infection occurred with a 4 h leaf wetness period. Increasing leaf wetness duration from 7 to 13 h lowered the temperature required for disease onset from 21 to 17 °C. More infection occurred with 13 compared to 10 h of leaf wetness at 17°C, and severity decreased for all leaf wetness periods at 29 compared to 27°C (Barrera et al., 2012). Boiteux et al. (1994) found that the optimum temperature range for infection of garlic by *S. vesicarium* was 22-26 °C.

At optimum temperature of 25/20 °C, as established in the *in vitro* and bioassay experiments, a minimum wetness period of 12 h is required to establish plant infection *in vivo* (Figure 5.7). However, Mwakutuya and Banniza (2010) reported a minimum wetness period of 6-8 h at optimum temperature for significant infection of lentils by *S. botryosum*. A wetness period of 6 h did not result in infection at any temperature in our *in vivo* experiments (data not presented) but did show a very low level of infection in the excised leaflet experiment. *In vitro* germination of conidia was merely 30% after 6 h of incubation compared to 70% germination after 12 h at the optimum temperature of 22.5 °C (Figure 5.4). The germ tube length after 6 h was 18 µm compared to 50 µm after 12 h incubation at the optimum temperature. *In vitro* experimental data on the mycelial growth suggests that the mycelial extension stops under dry conditions. These findings explain the lack of infection or appearance of symptoms after 6 h of leaf wetness *in vivo* (Figure 5.2, data not presented).
In conclusion, optimal conditions for sporulation of *S. botryosum* are diurnal light, moderate RH (85%) and slightly warmer (WA spring) temperature of 22.5 °C whereas optimum temperature and RH for mycelial growth are 27.5 °C and 95% respectively – slightly higher than for sporulation. The germination rate of *S. botryosum* was reduced at temperatures above or below 22.5 °C and moving to both extremes resulted in further lowering the germination. This suggests that temperature would be a major limiting factor for infection of NLL by GLS. Lower and higher temperatures such as 12.5 and 32.5 °C, respectively, can limit infection by lowering the sporulation and germination rate, inhibiting the formation of infection structures. Similar to the mycelial growth, a slightly higher temperature is optimal for germtube elongation, suggesting the greater role of day and night temperature in germination of conidia and penetration of the host plant surface. Presence of sufficient moisture is also crucial in ensuring establishment of infection. No lesions were observed in leaves subjected to RH below 90%. A longer wetness duration resulted in greater infection intensity at all temperatures. This result suggests that this disease will have a relatively narrow range of environments that are optimum for epidemic development. Integrating this information with known environments where lupins are produced could be a means to categorise regional and seasonal risk of disease outbreaks.

Overall, the current study provides empirical information about the influences of temperature and moisture on sporulation, germination and growth of the fungus and on establishment of infection in excised leaves and in intact plants. These results suggest the conditions for production of inoculum in NLL plantations and subsequent severity of GLS disease. This information can be used to establish critical parameters in development of a simulation disease progress model for GLS in NLL.
Figure 5.8: *In vitro* excised leaf experiment to test the effect of temperature and wetness duration on grey leaf spot symptom development in narrow-leafed lupins

Figure 5.9: No symptoms of the disease were observed on leaves of susceptible genotype WALAN2333 subjected to 0 hours of leaf wetness after inoculation
Figure 5.10: Grey leaf spot lesion on excised leaves of susceptible genotype WALAN2333 subjected to 6 hours of leaf wetness after inoculation

Figure 5.11: Grey leaf spot lesion on excised leaves of susceptible genotype WALAN2333 subjected to 48 hours of leaf wetness after inoculation
Figure 5.12: Plants of narrow-leafed lupin genotype WALAN2333 subjected to 48 hours of leaf wetness after inoculation in glasshouse experiment showing severe infection (right) compared to control plants subjected to 0 hours of leaf wetness (left)
CHAPTER 6

Host range of Stemphylium, overwintering of pathogen on debris and infection of lupin seed

Abstract

This study was carried out to investigate; (i) the ability of Stemphylium isolates from NLL to infect any of the commonly found plant species in the WA wheatbelt, (ii) the influence of environmental variables on release of spores from the infested trash, and (iii) the persistence of Stemphylium in NLL seed. The plants tested as alternate hosts were primarily other broadacre crop species within the WA farming system, including grain and pasture legumes. No leaf spot symptoms were recorded on any of the tested plants. For studies of spore release from trash, two week old seedlings of NLL genotypes; Mandelup (resistant) and WALAN2333 (susceptible) were placed over the infested trash weekly for 7 months. After each week the exposed seedlings were removed for incubation in the glasshouse and were observed for the development of disease symptoms. The number of spores (conidia and ascospores) of Stemphylium released from the trash was monitored during the same time period using spore traps that were refreshed at weekly intervals. GLS symptoms were first observed during the third week of May. The disease was recorded on trap plants continuously from that week until the last week of November. Highly significant ($P<0.01$) positive correlation was found between the number of conidia captured and the number of rain days in a week. Similarly, the correlation between disease severity and number of conidia was also found to be highly significant. Incidence of seed infection was investigated using two different fungal growth methods and seeds from diseased plants that were non-sterilised or surface sterilised. A 2% incidence of seed infection was recorded for non-sterilised seed whereas for surface sterilised seed the incidence was recorded to be 2.5%. However, the difference between mean incidences when compared by the Mann-Whitney U test was non-significant at $P = 0.05$.

6.1 Introduction

Narrow-leafed lupin (NLL; Lupinus angustifolius L.) is a leguminous crop grown in many countries either as grain legume or fodder. The majority of the Australian
production of NLL is from Western Australia (WA) where the sandy low nutrient soils across parts of the winter cropping zone preclude cultivation of other legume species but gain significant benefit from the inclusion of a leguminous component in the cropping rotation. Fungal pathogens have been one of the main constraints to production of NLL. Potentially damaging fungal foliar diseases occurring in WA are anthracnose (*Colletotrichum lupini*) and brown spot (*Pleiochaeta setosa*) (Thomas et al., 2008). During early utilisation of NLL in Western Australian cropping systems, a significant disease that concerned lupin growers was grey leaf spot (GLS) caused by *Stemphylium* spp. (Cowling and Gladstones, 2000). The pathogen causes lesions on leaves, stems and pods of infected plants and produces conidia as inoculum for secondary infections whereas primary inoculum comes from infected stubble. Initially the disease appears in basal region of infected plants and then spreads rapidly to healthy plants in the vicinity when suitable weather conditions prevail. Since the mid 1970’s, the introduction of a major resistance gene into the NLL breeding program has meant that the majority of released cultivars have been resistant (Cowling and Gladstones, 2000), and this disease has not been a constraint to NLL production in WA in that time. Recently however there has been a re-emergence of disease in breeding plots that coincides with increasing proportion of susceptible lines within the NLL breeding program, prompting a new research effort (Thomas et al., 2011).

*Stemphylium* spp. have a wide host range; besides lupins they also cause various foliar diseases in crops including lentil, spinach, onion, tomato, alfalfa, asparagus, peppers, pear and clover (Smith, 1940; Rotem et al., 1966; Rao and Pavgi, 1975; Lacy, 1982; Irwin, 1984; Koike et al., 2001; Kim et al., 2004; Taylor et al., 2007; Kohl et al., 2009). The ability of pathogens to infect a wide range of crops can reflect the ability to overwinter on alternate hosts in the absence of the primary host and provides better survival chances. Plant debris is considered the primary source of inoculum of *Stemphylium* in most host-plant species. After the overwintering period sexual fruiting structures of *Pleospora* are produced on debris of the plants that were infested by *Stemphylium* in the previous season (Johnson, 1990; Prados-Ligero et al., 1998; Basallote-Ureba et al., 1999; Kohl et al., 2009). In garlic, *P. herbarum* overwinters on diseased plant debris that provides primary source of inoculum in subsequent leaf spot epidemics (Basallote-Ureba et al., 1999). On alfalfa, *S. botryosum* has been reported to
overwinter on dead stems and leaves (Flint and Clark, 1981). In asparagus, ascospores from overwintering pseudothecia act as source of inoculum for infection of spears (Elmer et al., 1996). Infection of S. vesicarium on pear increased every year due to accumulation of inoculum in plant debris (Polfliet, 2002). In lupins, diseased plant debris is considered to play an important role in the early infection by Stemphylium (Thomas et al., 2011). However, the environmental conditions under which the spores are released into the air from the debris have not been determined. The influence of weather variables on the role of debris to act as a source of primary inoculum has not been explored. Furthermore, no previous studies have been carried out to investigate the virulence of Stemphylium isolates from NLL on commonly grown plant species in WA and the extent of field persistence is unknown.

Infected seed is an important means of transmission of seed borne pathogens and also can serve as a primary source of inoculum early in the season. Stemphylium has been reported to persist on onion, spinach and lentil seed (Aveling et al., 1993; Hernandez-Perez, 2006; Wunsch, 2013). The prevalence of Stemphylium on or within NLL seed has not been investigated. The available evidence from other host species indicates that NLL seed should be examined as a source of Stemphylium infection in any disease risk analysis.

The objectives of the current study were; (i) to investigate the ability of Stemphylium isolates from NLL to infect any of the commonly found plant species in WA wheatbelt, (ii) to study the role of infested plant debris in development of GLS throughout the NLL growing season and correlate with prevailing environmental conditions, and (iii) to investigate the persistence of Stemphylium in NLL seed.

6.2 Materials and methods

6.2.1 Alternate host test

6.2.1.1 Inoculum preparation

Two isolates of Stemphylium botryosum, WAC12986 and WAC13136 (Thomas et al., 2011), were used in this study. Both were obtained from the Western Australian Culture Collection. Fresh cultures were prepared by growing the isolates on 20% V8 agar medium for 14 days at 22 ± 2 °C and 12 h light/dark cycle (Everts and Armentrout, 2001). For inoculum preparation spores were harvested in 1% Tween-20 solution and
the number of spores per ml of solution was estimated by triplicate haemocytometer counts. Final concentration was adjusted to $1.50 \times 10^6$ spores/ml.

### 6.2.1.2 Plant material

Different cultivars of the following plant species – commonly grown in WA – were tested as host of NLL isolates of *Stemphylium*; chickpea (Tyson, Sona), field pea (Parafield, Dundale), faba bean (Fior), lentil (Sunny), canola (Bravo, Tanami), wheat (Calingiri, Wyalkatchem), barley (Buloke, Baudin), yellow serradella (Santorini), sub-clover (Dalkeith), strand medic (Angel), wild turnip, sandplain blue lupin (*Lupinus cosentini*), white lupin (*L. albus*) and yellow lupin (*L. luteus*). NLL genotypes, WALAN2333 and Mandelup were included in the experiment as positive and negative control hosts. Seeds of each plant species were planted into 14 cm diameter plastic pots containing 2 L of potting mixture. Three replications of each cultivar were arranged in a completely randomised design. The experiment was conducted in a glasshouse maintained at 20±2 °C ambient temperature.

### 6.2.1.3 Inoculation

Two week old seedlings were inoculated with a conidial suspension of each of the isolates, separately. Plants were sprayed with the conidial suspension (1ml/seedling) using an airbrush sprayer (Paasche Airbrush Co. USA) operated with a BOC 8000 air regulator (BOC Gas and Gear Australia) at 0.1 MPa. Control pots were sprayed with sterilized water. Following inoculation, pots were placed in a misting chamber for 24 hours, and then placed on a glass-house bench under ambient temperature 20±2 °C and observed over a two week period for the development of disease symptoms. The experiment was repeated twice.

### 6.2.2 Role of plant debris in the disease carryover

#### 6.2.2.1 Inoculum source and trap plants

Infested debris consisting of lupin stems and leaves was produced during the 2010 cropping season. To produce infested trash, plants of susceptible NLL genotype WALAN2340 were inoculated three times with *Stemphylium* isolate WAC13136 during the growing season. Plants were sprayed to incipient runoff with the conidial suspension ($1.50 \times 10^6$ spores/ml) using an airbrush sprayer (Paasche Airbrush Co., Chicago, USA)
operated with a BOC 8000 air regulator (BOC Gas and Gear, Welshpool, Australia) at 0.1 MPa. The plants were grown in a shadehouse covered with fine insect proof mesh, resulting in higher humidity and temperature growing conditions than the outside environment. At maturity, stems and leaves from the infected plants were collected for use as trash in the following year.

Trash (2 kg) was evenly spread on nylon mesh cloth placed over the soil surface of a 1.5 m × 2 m plot during the first week of May, 2011. The plot was located at the South Perth experimental area of the Department of Agriculture and Food Western Australia (DAFWA). Trash was covered with aluminium wire mesh, which was securely pegged at the four corners to prevent trash being blown away by strong gusts of wind.

Seeds of two NLL genotypes, Mandelup (resistant) and WALAN2333 (susceptible), were planted into 14 cm diameter pots (four plants per pot) containing 2 L of potting mixture. Plants were grown in the glasshouse at ambient temperature 20±2 °C. Pots containing two week old seedlings were placed over the trash plot. Every week a new set of three pots with plants of both genotypes was placed over the trash plot. Control plants of both genotypes were placed over a control plot area that had clean nylon mesh cloth over the soil surface without any trash. Plants were removed from the trash and control plots after one week of exposure and incubated in a misting chamber for 24 hours. The misting chamber consisted of a closed transparent plastic cage placed in a metal tray filled with water to provide 100% RH. The plastic cage was fitted with Defensor 505 portable humidifiers (JS Humidifiers plc, West Sussex, UK) to maintain a wet environment. Observations of foliar spot symptoms were made one week after the start of incubation and disease severity was recorded.

6.2.2.2 Spore release from trash

The number of spores (conidia and ascospores) of Stemphylium released from the trash was continuously monitored using spore traps. Three passive wind vane spore traps (Lacey and West, 2007) were placed on three corners of the trash plot, with the collection orifice being ~25 cm above trash level. Microscope slides coated with a thin layer of petroleum jelly were placed vertically inside the spore traps. A new set of slides was placed inside the spore traps every week on the same day as a new set of plants was placed on the trash. Every week, slides were removed from the spore traps and washed with 20 ml of hot (maintained
at 70 °C using hot-water bath) 1% NaOCl solution. The number of *Stemphylium* spores in the suspension was then estimated using a haemocytometer. *Stemphylium* spores were identified according to the descriptions of Simmons (1967).

### 6.2.2.3 Influence of weather variables on spore density

Correlations between the weekly spore density data and the weather variables for the collection period (accumulated seven day rainfall, number of rain days in the week, mean maximum temperature and mean minimum temperature over the seven day period) were tested. The weather data were obtained from the DAFWA weather server for the South Perth site (station was sited approximately 500 m from the experimental site). Regression analysis of the severity of GLS symptoms and the aerial concentration of spores was carried out across the test period.

### 6.2.3 Persistence of the pathogen on seed

#### 6.2.3.1 Seed Material

Test seeds were produced in 2011-12 from susceptible NLL genotype WALAN2333. The seeds were harvested from a plot that had been inoculated with *Stemphylium* infested trash (produced in 2010). Pods were hand harvested at maturity from plants showing more than 90% GLS disease severity. Pods were then hand-threshed to obtain seeds. Any shriveled and deformed seeds were excluded from the test sample. Seeds from healthy/uninfested plots were also obtained to be used in the control treatment. Seeds thus obtained were stored at room temperature for one year.

#### 6.2.3.2 Seed germination test

One year old seed of WALAN 2333 from diseased and healthy plots was tested for germination. A total of 400 seeds (8 replications of 50 seeds) of each of diseased and healthy lots were used for germination testing. Seeds were placed on double layered sterile paper towel that was placed within sterile 30×10 cm white plastic trays and moistened with 10 ml of sterile deionized water. The trays were incubated at 22±2 °C with a 12 h light/dark cycle (near-UV and cool-white fluorescent light by day) for 14 days. After the incubation period, germination percentage was recorded.
6.2.3.3 Seed assay

Two types of seed assays were performed: surface-sterilized and non-surface-sterilized. Each assay consisted of four replications of 200 seeds. The seed for each replication was placed into a mesh tea strainer. For non-surface-sterilized assay, the tea strainer containing seeds was placed under running deionized water for 10 minutes. The seeds were then dried in a laminar flow hood on sterile paper towel. For surface sterilized assay, the tea strainer was placed in 150 ml of 1% NaOCl and shaken manually for one minute and then triple-rinsed in sterile deionized water.

For non-surface sterilized assay, the dried seeds were then placed on double layered sterile paper towel that was placed within sterile 30×10 cm white plastic trays and moistened with 10 ml of sterile deionized water. Using sterile forceps seeds were transferred onto the paper towel (50 seeds per tray) and the trays closed with clip-on transparent lids. The method was adopted from the International Seed Testing Association (ISTA) standard blotter method (Neergaard, 1977). The trays were incubated in the dark at 22±2 °C for 24 h to allow the seed to imbibe and then transferred to an incubator set at 22±2 °C with a 12 h light/dark cycle (near-UV and cool-white fluorescent light by day) for 10 days.

For surface sterilized assay, the dried seeds were placed in Petri plates containing 20% V8 agar medium (5 seeds per plate). The method was adopted from the ISTA standard agar-plate method (Neergaard, 1977). The plates were incubated at 22±2 °C with a 12 h light/dark cycle (near-UV and cool-white fluorescent light by day) for 10 days.

The seed from both tests were examined by microscope 10 days after incubation. *Stemphylium* spp. was identified based on conidial morphological characteristics. The experiment was repeated twice.

6.2.3.4 Isolation of Stemphylium from infected seeds

*Stemphylium* spp. was isolated from infected seeds after 10 days of incubation. Mycelial mass and spores from the seeds were streaked onto V8 agar medium in Petri plates using a sterile inoculation needle. Plates were incubated under ambient laboratory conditions (12 h light/dark cycle and 22 ± 2 °C) for 10 days.
6.2.3.5 Virulence of seed isolates

Eight isolates of Stemphylium selected at random from the infected seeds were tested in the glasshouse for virulence on each of three NLL genotypes: Mandelup (Resistant), Unicrop (Susceptible) and WALAN2333 (Susceptible). Inoculum was prepared from the isolates derived from the infected seed on V8 agar medium. Spore suspensions were prepared from 10 day old single spore cultures grown at 20±2 °C under a cool-white fluorescent light/dark cycle of 12 h. To test the virulence of the isolates, seeds of each genotype were planted into 14 cm diameter pots (four plants per pot) containing 2 L of potting mixture. Plants were grown in the glasshouse at an ambient temperature of 20±2 °C. Three pots for each genotype with two week old seedlings were inoculated with a conidial suspension of each of the isolates at a concentration of 1.5×10^5 spores/ml. Plants were sprayed to incipient runoff with the conidial suspension using an airbrush sprayer (Paasche Airbrush Co. USA) operated with BOC 8000 air regulator (BOC Gas and Gear Australia) at 0.1 MPa. Control pots were sprayed with sterilized water. Following inoculation, pots were placed in a misting chamber for 24 hours, and then placed on a glass-house bench. Glasshouse temperature was maintained at 20±2 °C. Plants were observed over a 10 days period for the development of disease symptoms. The experiment was repeated twice.

Virulence of the isolates was assessed on the basis of mean lesion sizes on five leaflets from each plant. Each Stemphylium isolate was classified into one of four groups: highly virulent (>5 mm), virulent (2-5 mm) moderately virulent (1-2 mm) and avirulent (no lesions).

6.2.3.6 Identification of re-isolated fungi

Two weeks after inoculation, four leaflets showing symptoms of the disease were removed from each plant. Leaflets were surface-sterilized in 1% NaOCl for 1 minute, triple-rinsed in sterile deionized water, dried on sterile paper towel in a laminar flow hood, and placed onto V8 agar medium in Petri plates. Leaflets were also cut from asymptomatic leaves of the control plants and plated onto V8 agar. Plates were incubated at 22±2 °C with a 12 h light/dark cycle (near-UV and cool-white fluorescent light by day) for 10 days. After 10 days of incubation, leaflets were examined.
microscopically for the identification of fungal growth. Identification was done based on the morphology of conidiophores and conidia (Simmons, 1967).

6.2.4 Data analysis

Relationship between mean disease severity on trap plants and various weather variables i.e. rain days per week, cumulative rainfall during the week, mean minimum temperature and mean maximum temperature was analysed by simple linear regression. Regression analysis of the disease severity and number of conidia detected during the seven-day period was also performed. The difference between detection frequencies of *Stemphylium* in surface-sterilized and non-surface-sterilized seed assays was analysed by Mann-Whitney U test.

6.3 Results

6.3.1 Alternate host test

For both the isolates, only NLL genotype WALAN2333 developed the characteristic disease symptoms. No symptoms were observed on any plant belonging to any of the other species. Similarly, there were no symptoms of the disease on resistant NLL genotype Mandelup.

6.3.2 Development of leaf spots in the trap plots

At no time did symptoms of GLS appear on plants of the resistant genotype Mandelup. Similarly, no symptoms were recorded on control plants of either WALAN2333 or Mandelup (placed over clean surface) throughout the experimental period (Figure 6.3). Trap plants were deployed in the first week of May. There were no symptoms of GLS observed after incubation of plants that were placed on the trash plot during the first and second week of May. GLS symptoms were first observed on plants of the susceptible genotype (WALAN2333) placed on the trash plot during the third week of May. Disease was evident at 20% severity or greater from week 3 of the experiment (3rd week of May) until the last week of November, when three consecutive weeks of no infection occurred. Disease severity was recorded as 60% in the third week of May and varied between 20% and 90% between the 3rd week of May and the 3rd week of November, with maximum disease severity observed during the third week of September (Figure 6.1A).
6.3.3 Spore release from trash and effect on disease severity

Ascospore density, as detected by the spore traps, indicated that ascospores were not released in a continuous manner but sporadically between May and September. The first detection was made in the third week of May and coincided with rainfall during the week. The second detection was in the first week of June whereas the third and fourth detections were in the last two weeks of July and also coincided with rainfall. The last detection of ascopores was in the first week of September.

Conidia were detected throughout the period of observation. The conidial release showed a strong correlation ($r=0.89$, $P<0.001$) with the number of rain days per week (Table 6.1). The highest concentration of conidia was recorded during the first week of September. Severity of disease on trap plants was correlated ($r=0.92$, $P<0.001$) with conidial density (Figure 6.2). The most severe disease occurred in the first and third weeks of September, coinciding with a high aerial concentration of conidia, frequent rainfall, and mean weekly maximum temperature of 21.5 °C. In four observation periods, infection occurred in trap plants without conidia being collected. In two of these periods, ascospores were observed, however in two weeks neither ascospores nor conidia were observed. No infection was evident in control plants during those weeks and so it is assumed that the collection efficiency of the spore traps was not sufficient or that prevailing weather conditions favoured spore movement to the side of the plot that lacked a trap.

Table 6.1: Linear correlation coefficients between weather variables and pathogen/disease parameters during 2 May to 11 December 2011

<table>
<thead>
<tr>
<th>Weather Variables</th>
<th>Ascospore Density</th>
<th>Conidial Density</th>
<th>Disease Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{min}}$</td>
<td>-0.17$^{\text{NS}}$</td>
<td>-0.29$^{\text{NS}}$</td>
<td>-0.47$^{**}$</td>
</tr>
<tr>
<td>$T_{\text{max}}$</td>
<td>-0.22$^{\text{NS}}$</td>
<td>-0.55$^{**}$</td>
<td>-0.69$^{**}$</td>
</tr>
<tr>
<td>RD</td>
<td>0.38$^*$</td>
<td>0.89$^{***}$</td>
<td>0.76$^{***}$</td>
</tr>
<tr>
<td>CRF</td>
<td>0.58$^{***}$</td>
<td>0.76$^{***}$</td>
<td>0.66$^{***}$</td>
</tr>
</tbody>
</table>

$T_{\text{min}}$ = Seven days mean of minimum temperature, $T_{\text{max}}$ = Seven days mean of maximum temperature, RD = Rain days in seven days period, CRF = Cumulative Rainfall of seven days period. $P<0.05$ ($*$), $P<0.01$ ($**$), $P<0.001$ ($***$)
CHAPTER 6

C

$\times 10^6$ Conidia

D

Rainy days per week

8 May 11  8 Jun 11  8 Jul 11  8 Aug 11  8 Sep 11  8 Oct 11  8 Nov 11  8 Dec 11

8 May 11  8 Jun 11  8 Jul 11  8 Aug 11  8 Sep 11  8 Oct 11  8 Nov 11  8 Dec 11
Figure 6.1: (A) Mean severity of grey leaf spot symptoms on WALAN2333 trap plants placed over infested debris, (B) Mean weekly ascospore density of *Pleospora* spp. recorded by passive wind vane spore traps, (C) Mean weekly conidial density of *Stemphylium* spp. recorded by passive wind vane spore traps, (D) Number of rain days per week (Rainfall ≥ 1 mm per day) during the experimental period, (E) Weekly cumulative rainfall (mm), and (F) weekly mean maximum and minimum temperatures (°C) during 2 May – 11 December 2011 at South Perth
6.3.4 Incidence of Stemphylium

Incidence of *Stemphylium* in seeds of NLL genotype WALAN2333 was recorded to be 2% in the surface-sterilized and 2.5% for the non-surface-sterilized treatments. There was no significant difference ($P>0.05$) between the mean incidence of *Stemphylium* for the treatments.

6.3.5 Virulence of the isolates

All the seed isolates obtained during the seed assays were found to be pathogenic, and were ranked as highly virulent on susceptible genotypes (Unicrop and WALAN2333). Plants of resistant variety Mandelup remained asymptomatic (Table 6.2).
6.3.6 Seed germination

Germination percentage of seeds from diseased and healthy plots were not significantly different, germination rate for both seed lots was 97-99%. After incubation, un-germinated seed from the diseased plots exhibited black fungal growth of *Stemphylium* hyphae.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Seed infection Incidence (%)</th>
<th>Virulence of isolates on WALAN2333 / Unicrop</th>
<th>Mandelup</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Sterilized</td>
<td>2.0</td>
<td>Highly virulent</td>
<td>Avirulent</td>
</tr>
<tr>
<td>Non-Surface Sterilized</td>
<td>2.5</td>
<td>Highly virulent</td>
<td>Avirulent</td>
</tr>
</tbody>
</table>

6.4 Discussion

How a disease is perpetuated between and within growing seasons will significantly influence the regularity with which it occurs and the time of introduction into the crop.

None of the potential alternate hosts tested showed any leaf spot symptoms of Stemphylium GLS. *Stemphylium* spp. associated with garlic and onion has been reported to be host non-specific and caused similar symptoms in closely related hosts (Boiteux et al., 1994; Basallote-Ureba et al., 1999). The plants tested as alternate hosts were primarily other broadacre crop species within the WA farming system, including grain and pasture legumes. The lack of disease on any of the tested plants due to *Stemphylium* spp. isolated from NLL indicates that the primary source of inoculum is likely to be the NLL itself. Of particular interest is the lack of infection on sandplain blue lupin (*L. cosentini*) that could have been assumed to be a most likely host of this disease. It acts as a host of other diseases of NLL, most notably anthracnose (*Colletotrichum lupini*) for which its widespread occurrence in a region with significant NLL production is a problem (Thomas, 2003).
Stemphylium conidia were captured above infected trash by the spore traps throughout the seven month observation period and trap plants also developed disease symptoms during this period. These results support the hypothesis that spores of *Stemphylium* developed on lupin debris from plants infected from previous crops constitute a significant source of inoculum for the GLS disease.

Rainfall is one of the environmental factors most directly related with the spore density in the air. All detections of ascospores coincided with frequent and considerable amount of rainfall during the week, however, there was significant rainfall during last week of June and first week of July and no ascospores were recorded for that period. The association between rainfall and ascospores release has been reported in *Stemphylium* during study of leaf blight disease of garlic (Prados-Ligero et al., 2003).

The results do not demonstrate a clear role of temperature in the release of conidia as there was a non-significant relationship with the density of conidia. Temperature also did not show any relationship with ascospore release (Figure 6.1). However, temperature was related to severity of infection and in the colonization processes, with an optimal range of 19-22 °C as expressed by the severity of disease symptoms in the trap plants (Figure 6.1).

The hypothesis that Stemphylium would survive in seed from an infected crop was supported by this study. The capacity for transfer of *Stemphylium* in NLL seed was demonstrated by detection of the fungus in seeds assayed in this study at an incidence of about 2%. Detection of the pathogen in equal frequencies from surface sterilized and non-surface-sterilized assays suggests that *Stemphylium* is internally seed-borne in NLL.

*Stemphylium* has not previously been reported on NLL seed. However, the results are consistent with reports of the seed borne nature of *Stemphylium* that has been reported in various other hosts such as onion, garlic and spinach (Aveling et al., 1993; Cedeno et al., 2003; du Toit and Hernandez-Perez, 2005). All isolates of *Stemphylium* obtained from the infested seeds were pathogenic, and were ranked as highly virulent on susceptible genotypes. In morphology, the isolates resembled WAC13136 –the isolate of fungus that was used to produce infested trash. The ability of *Stemphylium* to infect the seed inside infected pods of NLL is thus demonstrated following Koch’s postulate.
The lack of growth on other hosts begs the question of what was the original source of the *Stemphylium* now found in WA. Obviously the alternate host testing has not been exhaustive and so it may have been growing on a native species not tested, or it might have been associated with seed material brought to WA. The finding that the pathogen can be seed borne suggests that it could have been brought to WA in imported seed lots. This could have occurred during the collaborative breeding program between WA Department of Agriculture and US Department of Agriculture or through other seed imports that occurred prior to the establishment of seed import biosecurity restrictions in 1996 (Nairn et al., 1996). Gladstones (1994) reports that GLS was not a problem for WA lupin industry when the collaborative breeding program began whereas Forbes et al. (1964) were already exploring the source of resistance against the disease in the USA. DNA analyses of the current isolates of *Stemphylium* from WA in comparison with the genetic diversity found in other locations around the globe will help to determine origins of each isolate.

In conclusion, this study has shown that to devise an effective management strategy for GLS disease, in addition to field sanitation, proper seed treatment of seed saved for the next season from a diseased crop should also be recommended for the susceptible genotypes of NLL.
Figure 6.3: Plant of narrow-leafed lupin genotype WALAN2333 showing grey leaf spot symptoms and defoliation were placed over plot with infested trash (label “T”). Asymptomatic healthy plants were placed over control plot without any trash (label “C”).
CHAPTER 7

Risk model for grey leaf spot disease of lupins in Western Australia

Abstract

A predictive risk model based on data collected from laboratory and field studies was developed for Stemphylium grey leaf spot of lupins and named Grey Leaf Spot of Lupins Risk (GLSLR) model. The GLSLR model predicts level of risk of the disease at 17 locations in WA based on cumulative disease index of severity of GLS disease on NLL leaves and summarises the extent of favourable temperatures and leaf wetness. Model predicted cumulative disease index and observed disease intensity were compared using a logistic regression model and the difference was found to be non-significant ($P>0.05$). Based on the risk categories assigned to sixteen sites across eight lupin growing zones of WA, a risk map was prepared as an outcome of the model. High to medium risk of the disease has been predicted for northern part of the high rainfall central (northern part of Zone 4) and northern (Zone 1) lupin zones of WA. Medium rainfall northern (Zone 2) and central (Zone 5) zones are predicted to have medium to low risk of the disease. The first results are promising, although the model still needs improvements.

7.1 Introduction

The prediction of plant disease risk helps in better managing disease. Theoretical models that predict risk of plant diseases are a useful outcome of epidemiological studies. Most of these models use weather events to predict risk of plant disease epidemics. Climate data are thus used to find the favourable periods for disease development to help devise disease management strategies which limit multiplication (Jabrzemski and Sutherland, 2006).

Host resistance to a disease is the main strategy to avoid rampant disease development and minimize yield loss. However, when this solution is not available, disease risk models provide essential information to deploy other methods of avoiding the epidemic (Moschini and Pérez, 1999). Risk models are found to be crucial for effective and economical application of fungicides to reduce crop losses resulting from virulent isolates of the pathogen (Saari and Prescott, 1985; Eversmeyer and Kramer, 1992). In
the case of narrow-leafed lupins (NLL), Diggle et al. (2002) have produced a model for predicting the risk of anthracnose disease development in Australian conditions. However, a model to predict the risk of *Stemphylium* grey leaf spot (GLS) disease of NLL has not been developed in Australia or elsewhere; this is of increasing importance as lines within the national NLL breeding program lack GLS resistance.

The effects of temperature and leaf wetness duration on the development of various plant diseases have been studied and mathematical models have been developed to predict the infection of healthy plants (Lalancette et al., 1988; Carisse and Kushalappa, 1990; Broome et al., 1995; Montesinos et al., 1995).

From the experience with other diseases, understanding the epidemiology of *Stemphylium* GLS disease of lupins and formulation of a risk prediction model incorporating location-specific weather conditions, will help in better managing the disease. Development of a disease risk model is also important due to limitations of resources and time to investigate the epidemiology of GLS for all growing environments. Thus, parameters derived from quantitative epidemiology under limited environmental conditions built into a model will provide substantial understanding of the epidemic risk in most of the growing areas.

A risk model of GLS disease, based on understanding the relationship between the disease and environment, will offer a more informed management approach for farmers and also help lupin breeders to plan and release varieties with appropriate resistance in high risk regions. Therefore, the main objective of this research was to develop a disease risk model, driven by weather parameters, for lupin growing regions of WA.

### 7.2 Materials and methods

#### 7.2.1 Description of the model

A predictive risk model based on data collected from laboratory and field studies (Chapters 3-6) was developed for *Stemphylium* grey leaf spot of lupins and named Grey Leaf Spot of Lupins Risk (GLSLR) model.

Plant debris is the primary source of inoculum of *Stemphylium* as established by inoculum treatment and trap plant experiments (Chapters 4 and 6). Under wet conditions conidia are released from the debris. Favourable conditions i.e. temperatures
of 10-35 °C with optimum 22.5 °C and relative humidity of > 95% are also required for the fungus to penetrate into and infect host tissues, to cause disease (Chapter 5).

The GLSLR model predicts level of risk of the disease at 17 locations in WA based on cumulative disease index of severity of GLS disease on NLL leaves and summarises the extent of favourable temperatures and leaf wetness. The model assumes that the primary disease inoculum is present in the field, in the form of Stemphylium-infested plant debris from the previous season, and infection begins at the onset of favourable weather conditions. The model also assumes that Stemphylium conidia require at least 12 h of leaf wetness to infect a susceptible NLL plant (5.3.4 and 5.3.5).

As leaf wetness data for crops in the field were not available, this is represented in the model in a simplified way by using rainfall data. Under Western Australian conditions rainfall of 0.1 mm in each hourly period provides a leaf wetness of 4 hours, and 1 mm rainfall per day provides sufficient leaf wetness in NLL to meet the leaf wetness criterion (Diggle et al., 2002).

7.2.2 Meteorological data

Meteorological data for three years (2010-2012) were obtained from the weather server of the Department of Agriculture and Food WA for 17 weather stations across the agricultural region of WA. The data included the following environmental variables that were utilized in estimating predicted disease severity: (i) daily minimum temperature, (ii) daily maximum temperature and (iii) daily rainfall.

7.2.3 Model development

The model algorithm consists of four principal relationships:

- Disease infection based on the number of rain days.
- Disease development determined by mean daily temperature
- Determining the percentage disease through combining the above two relationships
- Determining the disease index by comparing the above percentage with the maximum possible disease percentage under the most favourable conditions
7.3 Results

7.3.1 Disease progression with rainfall frequency and temperature fluctuation

A linear relationship between disease severity and density of conidia was previously established using trap plants. The density of conidia also showed a linear relationship with the number of rain days per week (Chapter 6) thus a direct relationship between disease severity and number of rain days per week was estimated. Relationship between rain days per week and disease severity was denoted as rain-disease (RD) value. The model assumes that one mm rainfall in a 24 h period is sufficient to provide required adequate wetness for germination of conidia (Diggle et al., 2002). The RD value based on number of rain days in a week (days with >1 mm rainfall) was estimated as a linear regression of disease severity against the number of days on which crop conditions are met with the favourable conditions for conidial germination and infection. If the rainfall threshold of one mm is met, the GLSLR model begins the estimation of RD value for the following day.
Figure 7.1: Effect of number of rain days (>1mm Rainfall per day) per week on disease severity in trap plant placed over infested trash for one week period (Chapter 6)

The following simple linear regression equation (Equation 7.1), representing the relationship between disease severity and rain days, was used to estimate RD value.

\[
RD \text{ value} = 17.558 + 10.313 \times \text{Number of rain days per week}
\]

**Equation 7.1**

After successful germination of conidia, the initial infection process requires favourable temperatures between 10 °C and 35 °C. This relationship between temperature and disease severity was established through laboratory studies (Chapter 5). If the NLL crop experiences favourable temperature (10-35 °C) following a rainy day, the GLSLR estimates temperature-disease (TD) value using Equation 7.2.
Figure 7.2: Effect of temperature on disease severity in excised leaves of narrow-leafed lupin genotype WALAN2333 after 48 hours of incubation (Chapter 5)

\[ TD \text{ value} = -35.264 + 11.074 \times (\text{Mean Daily Temp}) - 0.2381 \times (\text{Mean Daily Temp})^2 \]

**Equation 7.2**

The rain-temperature-disease (RTD) value is determined as the product of RD and TD values. Where the mean daily temperature is outside the favourable range, the model assumes there was no infection establishment and the RTD value is zero. RTD values for individual days of a week are added together to get RTD values per week. A maximum RTD value per week (58654.57) was estimated for an assumed the most favourable week having seven rain days and all days with an optimum daily mean temperature of 22.5 °C. Percentage disease (PD) value was calculated by converting the weekly RTD values to percentage of the maximum weekly RTD value (58654.57). Figure 7.3 shows weekly PD values for three years (2010-2012) at four sites across WA wheatbelt. Badgingarra and South Perth show higher PD values compared to Katanning (which has relatively cooler temperatures) and Merredin (with relatively fewer rain days).

PD values for all weeks of a growing season (1st of May to 1st of October) were added together to produce a cumulative disease (CD) value. The GLSLR model assumes that
maximum CD value is 500. Cumulative disease index (CDI) was calculated for all sites as a ratio of maximum CD value. CDI for various sites across WA wheatbelt is given in Table 7.1.
Figure 7.3: Graphs of percent disease values of grey leaf spot severity over the lupin growing seasons from 2010 to 2012 based on mean temperature and number of rain days per week. Panels A to D provide the data for the 4 weather station data set locations.

Table 7.1: Cumulative disease indices of grey leaf spot severity predicted by mean temperature and number of rain days per month for 17 weather station data set locations across WA wheatbelt

<table>
<thead>
<tr>
<th>Site</th>
<th>Year</th>
<th>Variable</th>
<th>May</th>
<th>Jun</th>
<th>Jul</th>
<th>Aug</th>
<th>Sep</th>
<th>CDI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Badgingarra</td>
<td>2010</td>
<td>R Days#</td>
<td>7</td>
<td>5</td>
<td>7</td>
<td>7</td>
<td>4</td>
<td>20.70</td>
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<td>Jun</td>
<td>Jul</td>
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* Rain days per month  
* Mean temperature (°C)

### 7.3.2 Comparison with field data

The application of GLSLR in field was evaluated at South Perth experimental plot during 2011 cropping season. Disease intensity data on GLS susceptible genotype WALAN2333 of NLL was obtained from trash only treatment (Chapter 4, Figure 4.3) and compared with the predicted value of cumulative disease index.

![Logistic regression curves](image)

**Figure 7.4:** Logistic regression curves of predicted cumulative disease index (May 21 to October 1) and observed disease intensity (June 11 to October 1). Solid lines represent the fitted curves and dotted lines indicate 95% prediction limits for the fitted curves.
Predicted cumulative disease index and observed disease intensity were compared using a logistic regression model. Figure 7.4 shows a comparison of the regression curves for both variables. There was over prediction of disease until 6th of August. After 6th of August the difference between the fitted curves of model predicted disease and observed disease intensity was non-significant ($P > 0.05$). Final disease intensity observed in the field plot experiment and model predicted cumulative disease index for October 1 also was not significantly different.

Figure 7.5: Relationship between predicted cumulative disease index and observed disease intensity at South Perth in 2011 on WALAN 2333 plants infested by trash application

7.3.3 Use of model to predict disease risk

Non-significant difference was found between the model-predicted cumulative disease index and final disease intensity of GLS observed at South Perth on 1st of October 2011. The cumulative disease indices produced by the model were thus used to predict risk of GLS across the WA wheatbelt.
Risk categories were assigned based on CDI values. The CDI value for South Perth in 2010 was 26.17 but no disease was observed at South Perth experimental plots treated with infested trash during that year. However, at Eradu in 2011 a virulent isolate of *Stemphylium* was found and the CDI value for Eradu in 2011 was 36.26. It was thus assumed that a critical value of CDI for appearance of disease symptoms is between these two values (26.70 and 36.26) and thus CDI value of 33 was considered as critical value (no sensitivity analysis has been undertaken for this critical value). Therefore, CDI value of more than 33 constituted a risk index (RI) of 1 and less than 33 CDI value was assigned an RI of 0. The cumulative RI over the three years of this study was used to categorise different sites into high (sum = 3), medium (2), low (1) and no risk (0) areas (Table 7.2).

Table 7.2: Disease risk based on cumulative disease index (CDI) for three years (2010-12) for various regions across WA wheatbelt

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<tr>
<th>Town</th>
<th>GLS Risk Index</th>
<th></th>
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<td>1</td>
<td>1</td>
<td>2</td>
<td>Medium</td>
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</table>

<sup>a</sup> More than 33% CDI =1, Less than 33% CDI=0

<sup>b</sup> Cumulative value of; 3 = High, 2 = Medium, 1 = Low and 0 = No risk
7.3.4 Grey leaf spot risk map for Western Australia

Based on the risk categories assigned to sixteen sites across eight lupin growing zones of WA, a risk map was prepared (Figure 7.6) as an outcome of the model.

High to medium risk of the disease has been predicted for northern part of the high rainfall central (northern part of Zone 4) and northern (Zone 1) lupin zones of WA. Medium rainfall northern (Zone 2) and central (Zone 5) zones are predicted to have medium to low risk of the disease. The GLSLR model predicted no risk of the disease in the southern part of the high rainfall great southern (southern part of Zone 4), medium
7.4 Discussion

This study was conducted to estimate the risk of GLS to lupins in WA. Since GLS, like other plant diseases, is driven by weather conditions, a simple weather-based model was developed to meet the objective of the study. The GLSLR model predicts level of risk of the disease at 17 locations in WA based on cumulative disease index of severity of GLS disease on NLL leaves and summarises the extent of favourable temperatures and leaf wetness. The principle of “favourable condition” for disease progression has been widely used in modelling other crop diseases, such as ascochyta blight (Didymella rabiei) in chickpea (Cicer arietinum) (Shtienberg et al., 2005), blackleg (Leptosphaeria maculans; L. biglobosa) in canola (Brassica napus) (Salam et al., 2003; Salam et al., 2007), and ascochyta or blackspot (Didymella pinodes) in field pea (Pisum sativum) (Salam et al., 2011).

In our study, weather parameters i.e. rain days per week and mean daily temperature were used to predict the development of GLS during the cropping season. Progression of the disease was explained by logistic growth model and compared with the observed disease intensity in the field experiment. The logistic function of disease development has been used in many reports (Lalancette and Hickey, 1984; Correll et al., 1988; Rossi and Giosuè, 2003; Jeger, 2004). Comparison of the predicted disease progression with the natural disease development in the field was highly encouraging. The cumulative disease indices produced by the model were used to predict risk of GLS across the WA wheatbelt because of non-significant difference between the model-predicted cumulative disease index and final disease intensity of GLS.

Risk categories were assigned based on CDI values. A risk map was prepared as an outcome of the model. No risk of disease in high rainfall southern zones was predicted due to lack of favourable temperatures for the disease as daily mean temperatures in this zone rarely reach the favourable temperature range during winter season. No disease was observed in susceptible experimental genotypes of NLL during 2011 survey of great southern zone (Chapter 3). Eastern lupin zones don’t received sufficient rainfall during the season to sustain the growth of the fungus and development of the disease.
These finding agree with the observations of Gladstones “A major disease that concerned lupin growers in WA in the early 1970s was GLS, which attacked *L. angustifolius* varieties as they approached maturity in high rainfall regions” (Cowling and Gladstones, 2000).

This outcome suggests that the model is a useful preliminary tool. Further refinement by examining disease incidence at multiple locations across the state will be required. As well, the model can be extended to predict the likely impact on this disease of a changing climate or for lupin production in other regions both within Australia and internationally. This will help to provide an indication to lupin breeders in those regions as to the importance of maintaining genetic resistance to GLS in their breeding germplasm.
CHAPTER 8

General Discussion

The current study was conducted to achieve the following objectives;

1. To identify the pathogen associated with grey leaf spot (GLS) disease in Western Australia (WA) by comparing: the morphological features; growth characteristics; a portion of the DNA sequence of isolates of Stemphylium spp. collected from symptomatic tissue on lupins in the WA agricultural region
2. To determine the economic significance of the disease by examining potential yield losses caused by the pathogen
3. To determine environmental conditions favourable for the disease, and the spread and survival of the pathogen
4. To develop a weather-driven epidemic risk model for lupin producing regions of WA

The objectives of this study were achieved as described in Chapter 3 (Objective 1); Chapter 4 (Objective 2); Chapters 5-6 (Objective 3); Chapter 7 (Objective 4).

Four species of Stemphylium (S. solani, S. botryosum, S. loti and S. vesicarium) have been reported to cause GLS disease in lupins across various parts of the world where lupins are cultivated (Wells et al., 1956; Edwardson et al., 1961; Wells et al., 1961; 1962; Wells et al., 1971; Gladstones, 1977). All of them cause similar symptoms. Three (S. solani, S. botryosum and S. vesicarium) of these species were identified from WA in this study as the cause of the disease. Irwin et al. (1986) identified and morphologically characterized Stemphylium spp. collected from various hosts in Australia and reported different species causing the same disease in USA and Australia. However, virulence of the isolates appeared independent of the species designation. All virulent isolates of Stemphylium tested in this study caused similar disease symptoms regardless of their species designation.

Previously, S. vesicarium and S. botryosum have been considered to be the cause of GLS disease of lupins in WA (Gladstones, 1994; Thomas et al., 2011). However, no survey has been undertaken previously to ascertain all the species of the pathogen involved with the disease. Descriptive information available for the historic isolates of the pathogen from WA
lupins is limited and species designation is assumed to be based on morphological studies at the time of collection. As discussed in Chapter 3, surveys undertaken during 2009-11 demonstrated the presence of the pathogen in the field. Three different species of *Stemphylium* were identified using morphological and molecular techniques. One of the isolates collected during the 2009 survey was found to be *S. solani*. This is the first Australian report of the association of *S. solani* with GLS disease.

Another important aspect of the surveys undertaken during this study was that disease samples collected from blue lupin (*L. consentini*) were found to be not caused by *Stemphylium* spp. infection. Blue lupins grow wild through many northern parts of the WA grainbelt, often adjacent to narrow-leaved lupin (NLL) crops, and they are an important component of the epidemiology of another disease, lupin anthracnose, as they act as a reservoir of inoculum for this disease (Thomas, 2003). As described in Chapter 6, alternate-host tests suggest that it is highly unlikely that blue lupin (or other lupin species) is host to any of the current collection of isolates/species of *Stemphylium* present in WA. This is an important finding, in that it suggests that the occurrence of disease will be wholly related to cropping of NLL.

The detection of virulent *Stemphylium* isolates from the field suggests that the disease remains present within the WA environment and could therefore pose a major threat to susceptible cultivars of NLL. Edwardson et al. (1961) attributed forage and grain yield losses to the susceptibility of lupin cultivars to GLS. Resistance was introduced into WA varieties to limit disease development and limit yield losses (Gladstones, 1982) from this disease, and this resistance has been successful since the late 1970s (Thomas et al., 2011). However, due to the limited occurrence of this disease over the last four decades, screening of new NLL varieties for GLS resistance was not considered important (Thomas et al., 2011). As a result, susceptible cultivars such as PBA Gunyidi have recently been released for field plantation. This study has confirmed that re-emergence of the disease is a consequence of not screening and selecting for the resistance, which has proven remarkably durable, and is not due to a mutation of the pathogen to overcome the resistance character (Chapter 3). This research has demonstrated that the resistance present in cultivars such as Mandelup is effective against all virulent isolates collected, regardless of the species. It has also demonstrated the importance of maintaining resistance within the NLL breeding program gene pool (Chapter 4).
As described in Chapter 4, the effect of NLL genotype susceptibility, isolate virulence and primary inoculum source, have been shown to account for different rates of disease progress and subsequent effect of disease on grain yield. The study suggests that under favourable environmental conditions with high inoculum pressure the disease can cause up to 60% loss in yield of susceptible NLL in WA. The pathogen survives on diseased plant debris in the field and the infested trash is the principal source of primary inoculum (Chapter 4 and 6). In other hosts, after the overwintering period sexual fruiting structures of Pleospora or conidia of Stemphylium are produced on debris of the plants infested by Stemphylium (Johnson, 1990; Prados-Ligero et al., 1998; Basallote-Ureba et al., 1999; Kohl et al., 2009). In this study both ascospores and conidia were produced on infested trash which served as a source of primary inoculum infecting NLL seedlings. The presence of trash infested with a virulent isolate and susceptible genotypes in the cropping system will be the most important contributing factors in the development and progress of the disease. In their presence, a favourable environment is needed to initiate the infection. As discussed earlier, virulent isolates of the pathogen have been shown to be present in the WA environment and hence the only other factor that is to be considered is the nature of a favourable environment.

Environment plays an important role in plant disease generally, by influencing host susceptibility, pathogen infectivity and the host-pathogen interaction (Campbell and Madden, 1990; Agrios, 2005). Temperature and moisture have been demonstrated in other studies to be the primary environmental factors affecting mycelial growth, production and germination of Stemphylium conidia, and development of disease on various hosts (Bashi and Rotem, 1974; Emery and English, 1994; Basallote-Ureba et al., 1999; Kim et al., 2004; Mwakutuya and Banniza, 2010).

This study determined that optimum conditions for sporulation of the pathogen are diurnal light, moderate RH (85%) and average temperature of 22.5 °C whereas optimum temperature and RH for mycelial growth are 27.5 °C and 95% respectively – slightly higher than for sporulation. The germination rate of S. botryosum was greatest at 22.5 °C and reduced significantly at temperatures either above or below this optimum (Chapter 5). Similar to the mycelial growth, slightly higher temperature is optimum for germtube elongation suggesting the greater role of day and night temperature in germination of conidia and penetration of the host plant surface. Temperatures either lower or higher than
the optimum range, such as below 20 °C or above 30 °C, respectively, can limit infection by lowering the germination rate of conidia and reducing the rate of mycelial growth therefore inhibiting the formation of infection structures. Temperature is a major limiting factor for infection of NLL by GLS; as was reflected in the controlled environment experiments where deviation of temperature from the optimum, 20/25 °C significantly reduced lesion development. This suggests that the cool winter temperatures that occur in some areas of the WA grainbelt would limit infection and subsequent sporulation of the pathogen, limiting the rate of epidemic development.

In addition to temperature, moisture is critical in production of inoculum and for establishment of infection. This study indicates that the release of spores from infested trash was strongly correlated with rainfall. Relative humidity greater than 90% or periods of leaf wetness of 6-12 h were crucial in ensuring establishment of infection. Longer wetness duration resulted in greater infection intensity at all temperatures (Chapter 5), although greater wetness periods were required at temperatures below the optimum to achieve similar rates of conidial germination. These data suggest that this disease has a relatively narrow range of environments that favour epidemic development and that infection within the field will be closely related to frequency and periods of rainfall providing adequate leaf wetness.

The importance of weather conditions for disease development was clearly demonstrated at the South Perth site over two years of this study. In 2010, despite the presence of susceptible genotypes and infested trash, GLS epidemic did not develop. However in 2011, a season with significantly greater rainfall, similar experimental treatments resulted in significant disease development and yield losses were measured.

As well as information for the current season, the study provides empirical information about the conditions for production of inoculum in NLL fields and the persistence of Stemphylium in trash and seed contributing to infection in a subsequent season. This information can be used as a starting point to establish the critical parameters for a simulation disease progress model for GLS in NLL. In the final stage of this study, an attempt was made to integrate the detailed information about epidemiological parameters that were generated by this study, with the information about known environments where lupins are produced, to categorise regional and seasonal risk of disease outbreaks (Chapter 7).
This risk assessment model indicates that significant regional and seasonal variation is evident in the risk of GLS occurrence. Using the weather data of the three years of the survey period, the risk assessment model suggests that the coastal strip between Perth and Geraldton has the greatest annual risk of GLS epidemics. This region has warmer winter temperatures and sufficient rainfall to support GLS epidemic development. On the basis of this risk assessment tool, it is recommended that NLL varieties developed for this high risk region must be resistant to GLS. However, on the basis of cooler temperatures or insufficient rainfall, susceptible cultivars could conceivably be deployed into the central, eastern and southern agricultural regions of WA with minimal risk of GLS outbreaks. Using this tool, assessment of risk in lupin production zones across Australia and assessments of the impact of climate change could be carried out to predict future importance of GLS resistance to the NLL breeding program.

The current study demonstrated the presence of diversity in the species of *Stemphylium* spp. infecting NLL. However, because the number of isolates of *Stemphylium* and genotypes of NLL used in the virulence study were limited, a more detailed study on the diversity of *Stemphylium* spp. and their interaction with varieties with a range of genetic backgrounds is warranted. Investigating the origin and distribution of *Stemphylium* and susceptibility of the current suite of NLL cultivars in production will assist in devising strategies to reduce the primary inoculum of the pathogen and its subsequent spread to vulnerable populations of NLL and potentially to other lupin crops.
REFERENCES


REFERENCES


Gladstones, J.S., 1982. Structure of the narrow-leafed lupin breeding programme in the Western Australian Department of Agriculture. Department of Agriculture, Western Australia, South Perth.
REFERENCES


Hintze, J., 2013. NCSS 9. NCSS, LLC, Kaysville, Utah, USA.


References


REFERENCES


Li, X., 2012. Development of molecular markers linked to genes of important agronomic traits for marker-assisted selection in lupin (Lupinus angustifolius L.) breeding. School of Plant Biology. The University of Western Australia, Crawley, Western Australia.


REFERENCES

Mwakutuya, E., 2006. Epidemiology of Stemphylium blight on lentil (*Lens culinaris*) in Saskatchewan, Department of Plant Sciences. University of Saskatchewan, Saskatoon.


Simmons, E.G., 1985. Perfect states of Stemphylium II. Sydowia 38, 284-293.

REFERENCES


REFERENCES


## Appendix

**Different narrow-leafed lupin genotypes used in experiments during this research project**

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<td>Chapter 6</td>
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