Epidemiology of blackleg disease of canola, caused by *Leptosphaeria maculans*

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Abstract

Blackleg, caused by *L. maculans*, is of major economic importance in the canola growing areas of Australia. The aim of this research was to gain information about factors affecting the epidemiology of blackleg in south-eastern Australia. The effect of temperature on a number of aspects of the life cycle of *L. maculans* was studied in a controlled environment. Germination of ascospores was greater on agar and cotyledons than on leaves, on susceptible cultivars than resistant cultivars, and at 15 and 20°C than at 5 and 10°C. Elongation of germ tubes was greater at higher than lower temperatures and generally greater on cotyledons than on leaves of the cultivars after incubation for 24 h at 10-20°C. Temperature had a greater influence than plant organ or cultivar on ascospore germination and the elongation of germ tubes.

Temperature, wetness duration, cultivar and leaf position (leaves 1-3) influenced the incubation period of *L. maculans* on canola. Ascospores infected six canola cultivars over 10-20°C following leaf wetness duration of 16-72 h. Incubation period (from inoculation to the appearance of first lesions) was generally shorter at higher temperatures and following longer wetness periods. Incubation period decreased with increasing leaf age, regardless of the blackleg-resistance rating of the cultivars. Pseudothecia developed on naturally infested canola stubble incubated at 5-20°C under continuous wetness and a 12 h photoperiod. The time taken for pseudothecia to mature ranged from 58.3 days at 5°C to 22.2 days at 15°C. Fewer pseudothecia developed on stubble incubated in darkness at 15°C than in light. Pseudothecia took longer to mature when wetness was interrupted than continuous. More ascospores were released at 20°C than 5-15°C, although peak sporulation occurred earlier at 5-10°C. Discharge of ascospores continued for 8 h at 5-10°C, for 10 h at 15°C, and for 12 h at 20°C.

The effect of burial of infested canola stubble in sand and field soil (in pots) on pathogen survival, pathogenicity and stubble-associated fungi was studied over 13 months in ambient conditions. The isolation frequency of *L. maculans* from stubble decreased by 63.3% after 13 months of burial, regardless of soil type. Although the frequency of isolation of *L. maculans* and *Alternaria* spp. from stubble decreased over time, that of *Stachybotrys chartarum*, *Fusarium* spp. and *Coprinus* sp. increased substantially over the sampling period. Stubble buried in field soil underwent more decay than in sand over the 13 months. Pseudothecium formation on buried stubble decreased with increasing duration of burial and ceased after 11 months in the field soil and 13 months in sand. The time required for pseudothecia to mature was 25 days for stubble retrieved after one month and 30 days when retrieved 10 months after burial, regardless of soil type. Ascospores from the stubble recovered from burial caused typical Phoma leaf spots on canola over the first 9 months of burial. Over the year, 16 genera of fungi and 12 colony types of bacteria were isolated from the field soil on peptone-rose bengal agar (PRA) and on crystal violet agar (CVA), respectively. Populations of fungi and bacteria isolated on soil-extract agar were markedly greater than those on PRA and on CVA, respectively.

Growth and sporulation of *L. maculans* on agar media and on infested canola stubble was affected by a variety of fungi obtained from stubble buried in soil or from field soil. Antagonistic activities observed included lysis, deformation, overgrowth and inhibition of *L. maculans* hyphae, and reduction of pseudothecium density on stubble to one-third or less than that on controls following inoculation with *F. equiseti*, *Gliocladium roseum*, *Trichoderma aureoviride*, *Sordaria* sp. and an unknown Coelomycete. *S. chartarum* and *Coprinus* sp. reduced the mass of canola stubble *in vitro*.

Findings will contribute to a better understanding of blackleg and to the development of more effective control measures.

Abbreviations

ANOVA	Analysis of variance
CFU	Colony forming units
CVA	Crystal violet agar
cv.	Cultivar
cvs.	Cultivars
DNA	Deoxyribonucleic acid
DV8A	Dilute vegetable juice agar
G–	Gram-negative bacteria
G+	Gram-positive bacteria
GS	Growth stage
LSD	Least significant difference
NA	Nutrient agar
NUV	Near ultra violet
PDA	Potato dextrose agar
PRA	Peptone-rose Bengal agar
RO	Reverse osmosis
SEA	Soil-extract agar
WA	Water agar

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Publications

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Chapter 1. Introduction and Literature review

1.1 Introduction

Leptosphaeria maculans (Desm.) Ces. Et de Not. (anamorph *Phoma lingam* (Tode: Fr.) Desm.) causes blackleg (stem canker) on canola (oilseed rape, *Brassica napus* L. var. *oleifera* (Metzer Sink.)). The disease is of major economic importance in the main canola growing areas of Australia, Canada and Europe. The most severe epidemics occur in Australia, where the disease curtailed development of the emerging canola industry in the early 1970s (Bokor *et al.*, 1975). The introduction of blackleg-resistant canola cultivars resulted in the increase of canola cultivation; however, the use of resistant cultivars as the only strategy to control blackleg resulted in epidemics of the disease in the areas sown to these cultivars (West *et al.*, 2001). Thus, the widespread cultivation of resistant cultivars imposed selection pressure on the pathogen for virulence towards those cultivars. Although many studies have been conducted on the disease, blackleg continues to threaten the viability of the Australian canola industry.

This review studies the history of canola and the effect that blackleg has had on the industry in Australia. The epidemiology of this disease will be studied by looking at the processes of infection, ascospore germination, pseudothecium maturation and ascospore discharge. The importance of understanding these aspects of the epidemiology of the disease to predict blackleg, using forecasting models, is demonstrated.

1.2 Background

1.2.1 Canola cultivation in Australia

Rapeseed (*Brassica napus* L. and *B. rapa* L.) has been cultivated for centuries in Europe and Asia (Downey, 1966). In Australia, rapeseed production began in the mid-1960s (Cutting, 1975) with cultivars introduced from Canada. The area sown increased rapidly through to the early 1970s when growers realised the potential of this alternative cash crop (Salisbury *et al.*, 1995). Oil was first extracted from rapeseed seed in Canada for human consumption in 1956. The term canola describes cultivars producing seed with less than 2% of the toxic fatty acid, erucic acid, and less than 40 μmol l^{-1} of total glucosinolates, whereas the term rapeseed refers to cultivars which produce seed with higher contents of erucic acid and glucosinolates (Lamont, 1991). Canola (*Brassica napus* L.) is now a major oilseed in Australia, with an estimated 1.7 million ha in 1999 (Anonymous, 1999). The first rapeseed breeding program in Australia began in Victoria, south-eastern Australia in 1970. The first Australian cultivars categorised as canola were Marnoo (Victoria), Wesbrook and Wesroona (Western Australia), released early in the 1980s. Due to the threat of blackleg epidemics to the industry, breeding programs concentrated on producing blackleg-resistant cultivars. Jumbuck (1982), Maluka and Shiralee (1987) were released in New South Wales, providing increased yield, quality and blackleg resistance. In 1988, the first *B. napus* hybrid canola, Hyola 30, was produced and was followed by Hyola 42 in 1991 (Colton & Potter, 1999).

1.2.2 History of blackleg disease

A saprophytic organism, *Sphaeria lingam*, was found on dead red cabbage stems (Tode, 1791). The same fungus was reported from living *Brassica oleracea* (cauliflower plants) and reclassified to the genus *Phoma* (Desmaziere, 1849). *P. lingam* (Tode ex Fr.) Desm. was later recognised as an important worldwide pathogen of crucifer crops (Punithalingam & Holliday, 1972). The sexual stage of *P. lingam* was first found in New Zealand (Anonymous, 1957) and confirmed as *Leptosphaeria maculans* (Desm.) Ces & De Not., a pseudothecial loculoascomycete (Punithalingam & Holliday, 1972). *L. maculans* attacks seedlings, cotyledons, leaves, stems, roots and pods of canola, and produces lesions and basal stem cankers or blackleg (Gabrielson, 1983).

Until 2001, strains of *L. maculans* had been classified into two broad subgroups or pathotypes. The pathotypes are visually indistinguishable in culture but can be differentiated on their ability to produce pigment in culture, colony growth rate, molecular characters and disease reactions on *B. napus* (Howlett *et al.*, 2001; Williams & Fitt, 1999). These two pathotypes have been termed A-group (highly virulent) and B-group (weakly virulent). The differences between these groups are so profound that the B-group strains are now known as another species, *L. biglobosa* (Shoemaker & Brun, 2001).

Epidemics of blackleg occurred in western and southern Australia in 1971 and a serious general epidemic in 1972 devastated many canola fields (Wood & Barbetti, 1977). Total loss of the crop due to seedling death is rare and yield losses at harvest are commonly <10%, however they can reach 30-50% (Barbetti & Khangura, 1999). Of course, the severity of epidemics differs

substantially between seasons, between areas and between crops. In the UK, estimates of losses from blackleg, using national disease survey data and a yield loss formula, were *ca* € 56M per season in harvest years 2000-2002 (Fitt *et al.*, 2006a). Findings in medium-high rainfall (550-650 mm) areas of southern New South Wales, Australia, suggested that for each 1% reduction in the incidence of blackleg lodging, yield increased by 5% (Kirkegaard *et al.*, 2006). In spite of the introduction of blackleg-resistant canola cultivars, the disease continues to threaten the ongoing viability of the canola industry (Khangura & Barbetti, 2001; Salisbury *et al.*, 1995). Therefore, integrated management strategies are needed to effectively control the disease in Australia.

1.2.3 Disease symptoms

Lesions on cotyledons and leaves are generally greyish in colour, and vary in size and shape (Gugel & Petrie, 1992). Numerous, black, pinhead-sized pycnidia are often observed in the dead leaf tissue at the centre of these lesions. Pycnidiospores are usually produced in cirrhi, appearing as pink or purple exudates. Stem lesions, or cankers, are similar in shape to leaf lesions and usually are sunken into the stem tissue with a purple or black border. Lesions can girdle the basal stem and cause the plants to lodge and die.

The fungus produces black, immersed to erumpent, globose (300-500 µm diameter), ostiolate pseudothecia on dead leaves, stem and roots of crucifer plants. Pseudoparaphyses are hyaline, septate and filiform. Asci are clavate to cylindrical, bitunicate, 80-125 × 15-22 µm and 8-spored. Ascospores are 5-septate, cylindrical to ellipsoidal, yellow to brown, 35-70 × 5-8 µm, guttulate with rounded ends (Figure 1.1). Pycnidia are globose (200-400 µm diameter) or sclerotioid (200-

500 µm across). Pycnidiospores are hyaline, shortly cylindrical, mostly straight, guttulate, unicellular, 3-5 × 1.5-2 µm (Punithalingam & Holliday, 1972).

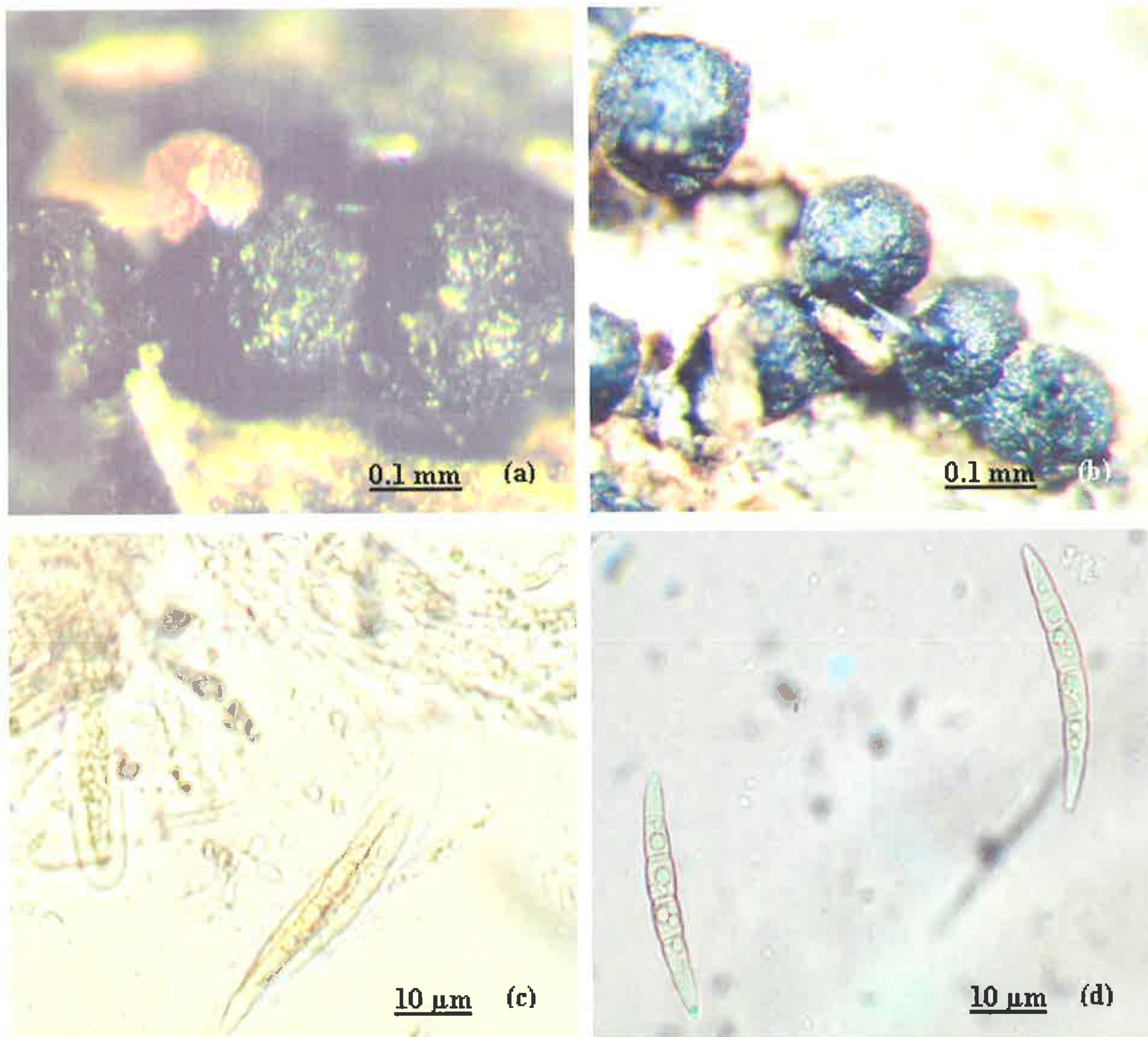


Figure 1.1. Microscopic morphology of *Leptosphaeria maculans*; (a) pycnidium with cirrhus, (b) pseudothecia, (c) asci and (d) ascospores.

1.3 Epidemiology of blackleg

1.3.1 Life cycle of *Leptosphaeria maculans*

An understanding of the life cycle of *L. maculans* allows us to manage canola crops with minimal exposure to the damaging effects of the pathogen. The life cycle is summarized in Figure 1.2.

In Australia, blackleg on canola is usually monocyclic (West *et al.*, 2001) and epidemics are initiated by sexual spores (ascospores) produced in pseudothecia on infected canola stubble (Barbetti, 1975; McGee, 1977). Ascospore release occurs after wetting by rain or even dew (McGee, 1977). The period of ascospore release varies from region to region but usually coincides with the presence of young, susceptible plants. In Australia, ascospores are first released from debris in May in response to winter rainfall, which is also required for seedling emergence (Bokor *et al.*, 1975).

Seedlings are invaded via stomata or wounds in the young leaves and cotyledons. Following initial infection of the leaf, *L. maculans* colonizes intercellular spaces between mesophyll cells, and grows down the petiole, mainly in xylem parenchyma and the cortex. The intercellular systemic phase of fungal growth is biotrophic and without visual symptoms (Hammond *et al.*, 1985). Behind the hyphal front the pathogen becomes necrotrophic and later produces pycnidia in the dead tissues (Hammond *et al.*, 1985). Pycnidiospores, which act as secondary inoculum, are spread by rain splash to other leaves and neighbouring plants.

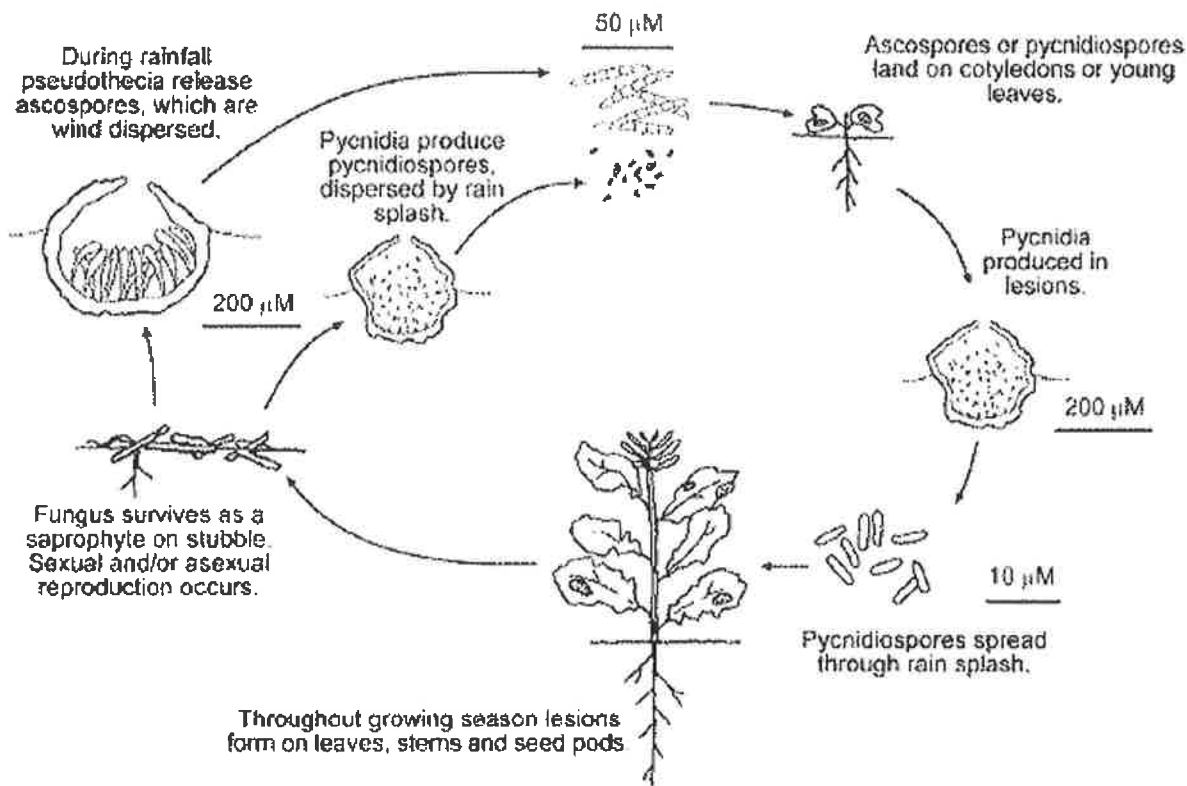


Figure 1.2. Life cycle of *Leptosphaeria maculans* on canola (Howlett *et al.*, 2001)

The cells of the stem cortex are finally killed by the pathogen, causing blackened cankers (blackleg), which can completely surround the base of the stem of canola. The process of producing stem cankers is also necrotrophic. Stem cankering is the major cause of yield loss associated with blackleg disease and severely affected plants lodge and die without producing seed (Howlett *et al.*, 2001).

1.3.2 Inoculum

1.3.2.1 Sources of inoculum

In Australia, primary inoculum of *L. maculans* occurs mainly in the form of air-borne ascospores produced in pseudothecia (McGee, 1977), which develop in canola stubble. *L. maculans* persists in a saprophytic mode on dead residual tissues in soil (Williams, 1992). The fungus can survive on canola stubble for at least 5 years (McGee, 1977). Canola stubble in Victoria, Australia, broke down in volume by 90% during the first year after harvest and liberated fewer ascospores in second year (Hall, 1992). Marcroft *et al.* (2003b) reported that the crucial factor in decrease in inoculum production in the field in southern Australia was the physical amount of residue persisting. This was supported by the observation in South Australia that *L. maculans* was not detected in soils 3 or more years after cropping when examined by a DNA-based assay. In addition, DNA of the fungus was found predominantly 0-5 cm from the soil surface, with very little from 5-15 cm (Sosnowski *et al.*, 2006).

Turkington *et al.* (2000b) also demonstrated that incorporation of stubble into soil increased decomposition, but this did not necessarily cause a decrease in potential inoculum (pycnidiospores) production. Higher pycnidiospore levels were observed in incorporated than surface-placed stubble, following 4 weeks incubation on vermiculite at 16°C. They believed that a reduction in the nutrient supply of the residue (as a growth medium) as a result of increased decomposition triggers the sporulation of most fungi, however, they did not study ascospore production on stubble. This finding was in agreement with an earlier study (Kharbanda & Ostashewski, 1997), which showed that blackleg-infested stubble buried in soil produced pseudothecia and ascospores of *L. maculans* more rapidly than residue left standing on the soil

surface. It is evident that the gradual accumulation of stubble on the soil surface enhances soil surface moisture levels (Steiner, 1994), which likely favour microbial activity and affect stubble decomposition and pathogen survival (Sutton & Vyn, 1990).

Survival of *L. maculans* in soil could be influenced by associated mycobiota on canola debris or by mycobiota of soil surrounding buried canola stem debris. Naturally occurring species of *Trichoderma* present in the rhizosphere are believed to produce some control of certain soil-borne pathogens (Papavizas, 1985). This finding was supported by the observation of Baird *et al.* (1999) that a decrease of isolation frequencies of *L. maculans* and all other fungi saprophytically present on buried canola stems corresponded with the increase in isolation frequencies of *Trichoderma* spp.

Although the importance of environment and soil factors in the production of inoculum and the survival of *L. maculans* persisting on residue is known, the influence of soil characteristics on pathogen survival is little understood. This project aims to examine the influence of different soil types on the survival of *L. maculans* on stubble collected from South Australian fields of canola.

Contaminated seed is likely to have spread blackleg to North America in the 1970s and is currently a major threat to spread the disease to China (West & Fitt, 2005). Although blackleg-infested seed may introduce *L. maculans* into an area, it is not generally an important source of inoculum (Gabrielson, 1983). For example, in Victoria, McGee (1977) found no relationship between disease incidence or severity and incidence of contaminated seed. In contrast, in Western Australia, a correlation between incidence of the disease and the incidence of contaminated seed was observed (Wood & Barbetti, 1977). Given that Wood and Barbetti (1977) performed their

study at locations remote from sources of ascospores in an unusual condition for disease development, findings that disease incidence is not related to seed contamination are likely to be valid in most regions.

Non-rape plants and their debris can also act as a reservoir and source of inoculum of *L. maculans*. A wide range of plants, mostly belonging to the Cruciferae and also the Compositae, Onagraceae and Gentianaceae, have been reported to be host plants of *L. maculans* (Gabrielson, 1983). In Australia, wild radish stubble in young canola fields could act as a significant source of inoculum of the fungus (Barbetti, 1978).

Infested soil is not considered a major source of inoculum, because *L. maculans* cannot persist for more than a few months in soil in the absence of host residues (Gabrielson, 1983).

1.3.2.2 Production of inoculum

Pycnidia of *L. maculans* develop rapidly on infected plant tissue in the field and in the laboratory. Although of relatively minor importance, pycnidiospores produced from primary leaf lesions may cause blackleg in suitable environmental conditions (Barbetti, 1976). In contrast, even small amounts of blackleg-infested residue can produce sufficient numbers of ascospores to spread the disease throughout a crop (Petrie, 1995b).

In Australia, the periods of ascospore production coincide with the susceptibility of crops to infection (McGee & Petrie, 1979). There was no difference between the timing of pseudothecium maturation on stubbles of different cultivars, including susceptible, slightly susceptible and

tolerant cultivars in France (Poisson & Pérès, 1999b), however stubble of resistant cultivars discharged fewer ascospores than susceptible cultivars in Australia (Marcroft *et al.*, 2004). Karbanda and Ostashewski (1997) observed clear differences in the timing of pseudothecium formation and ascospore discharge between different regions and concluded that environmental factors influence the differential development of pseudothecia. Understanding the environmental factors suitable for the production of pseudothecia (containing ascospores) allows us to predict the timing of presence of the inoculum in the field and to provide growers with effective recommendations for crop rotation and fungicide application.

The studies of Pérès *et al.* (1999) indicated that a mean temperature of 14°C and a high frequency of rainfall or 100% relative humidity provided the most suitable conditions for the appearance of pseudothecia. These epidemiological observations in the field were to be integrated to develop a model for forecasting pseudothecium maturation. A simple model (Bernard *et al.*, 1999) was developed to forecast the occurrence of mature pseudothecia capable of releasing ascospores, based on collection of biological data and weather data from six regions in France. Another model (Salam *et al.*, 2003a; Salam *et al.*, 2003b) was also developed for predicting the onset of pseudothecium maturity and seasonal ascospore discharge in relation to *L. maculans* in Western Australia. The model considered a combination of two weather parameters, daily average temperature and rainfall thresholds, to drive progress of pseudothecium maturity on stubble left from past crops. Each day was categorized as suitable or not suitable for progress of this maturity. The onset of pseudothecium maturity occurred after approximately 43 favourable days. The model satisfactorily predicted the timing of ascocarp maturation in 3 years of field observations in four regions of Western Australia. Furthermore, the relationship between the incidence of stem canker in summer and rainfall in the previous August, September and January

(Gladders & Symonds, 1995), used as the basis of an annual forecast in eastern England, provides indirect evidence that factors affecting pseudothecium maturation on stubble are crucial in determining the subsequent severity of blackleg (West *et al.*, 1999a). Therefore, understanding the factors affecting pseudothecium maturation on stubble in each area based on particular climatic factors is important not only to develop forecasting models but also to estimate the severity of the disease. The effect of environmental factors on differential stages of pseudothecia on stubble is little understood. This project aims to explore the influences of temperature, wetness conditions and darkness on formation of pseudothecia on stubble, collected from South Australian fields of canola, in a controlled environment.

1.3.2.3 Dissemination

Ascospores of *L. maculans* are air-borne and occur in a seasonal pattern (Petrie, 1995a). These are discharged during several months of the year but peak spore catches often occur in June, July and August (winter) in Australia (McGee, 1977). In addition, ascospore discharge maximal at a wind speed of 3 ms^{-1} (Kruger & Wittern, 1985) and rainfall of >1.0 mm (McGee, 1977). In Canada, it was reported that ascospore release was highly affected by rainfall, wind speed and wind direction (Guo *et al.*, 2003). An earlier study (Schramm & Hoffmann, 1991) showed that the number of ascospores discharged was influenced by rain (qualitatively) and temperature (quantitatively). According to Pérès and Poisson (1997), the first emissions of ascospores occurred 16-19 rain-days after harvest when the mean temperature dropped to 14°C. They applied a combination of the data of first spore release and weather conditions suitable for ascospore maturation to develop a preliminary model to predict blackleg and guide spray timing in France.

In order to develop similar forecasting model in South Australia, it is necessary to collect data for the influence of environmental factors on ascospore release.

There was no relationship between number of ascospores released and incidence of leaf lesions or severity of stem canker in Germany (Thurwachter *et al.*, 1999). However, Thurwachter *et al.* (1999) observed a higher disease incidence in years with an early spore release than a later spore release, relative to each site. In contrast, also in Germany, Kruse & Verreet (2005) reported a very close correlation was determined between ascospore release and leaf infection in autumn, September to November (Kruse & Verreet, 2005). The number of ascospores discharged from stubble in the field was related to blackleg severity in Western Australia (Wherrett *et al.*, 2004). Furthermore, in the UK and France, a large number of plants with leaf lesions was often observed prior to any main spore discharge (West *et al.*, 1999a; West *et al.*, 1999b), confirming the ability of the first ascospores released to produce a high incidence of leaf infection due to the susceptibility of the crop to infection in early growth stages.

Gradients of ascospore dissemination depend on the wind-speed, topography of the area and type of crop canopy, which filters ascospores from the air (West & Fitt, 2005). Ascospores are considered a risk to crops several kilometres away in Australia (Bokor *et al.*, 1975). However, the greatest risk of infection is within 500 m of the inoculum source (Barbetti & Khangura, 2000) and a separation distance of 400 m between canola stubble and new crop is recommended (Marcroft *et al.*, 2003b). Ascospores can remain viable for approximately 6 weeks, so a small number of spores are likely to travel long distances (Paul & Rawlinson, 1992).

1.3.3 Infection

Both ascospores and pycnidiospores adhere to the cotyledons and leaves of new crops and germinate in humid or wet conditions to produce the hyphae that cause infection. The ascospores of *L. maculans* germinate quickly. Many ascospores germinated after 8 h at 4-8°C, whereas pycnidiospores needed 24 h at 16°C to have similar development (Wittern & Kruger, 1985). Ascospores of both L. maculans and L. biglobosa germinated at 5-20°C on distilled water agar or detached canola leaves and the percentage of spores germinating increased with increasing temperature from 5°C to 20°C (Huang *et al.*, 2001). The two groups differed in germ tube length, average number of germ tubes and pattern of ascospore germination.

Germination of ascospores is an important stage before infection of leaves or cotyledons by *L. maculans* and forecasting models need to utilize accurate data on all stages of disease development, taking into account environmental conditions, canola cultivar and pathogen genotypes. There are many reports of the variability of *L. maculans* populations from different geographical locations (Barrins *et al.*, 2002; McGee & Petrie, 1978; Sosnowski *et al.*, 2001b), although isolates of the pathogen from seven sites in both eastern Australia and Western Australia showed a low degree of genetic differentiation (Barrins *et al.*, 2004). Furthermore, the influence of temperature on germination of ascospores of *L. maculans* on leaves of canola cultivars in Australia is not well understood. Therefore, this project will examine the effect of temperature on ascospore germination and hyphal growth from ascospores of *L. maculans,* collected from canola fields in South Australia.

A number of biological and environmental factors are known to influence the infection of canola leaves inoculated with *L. maculans*. In the UK, 4 h was the minimum wetness period required by ascospores of *L. maculans* to produce leaf lesions (Biddulph *et al.*, 1999a) and most lesions appeared following 48 h leaf wetness at 20°C (Biddulph *et al.*, 1999b). Leaf lesions appeared after 5 days at 20°C and after 2 weeks at 8°C, and leaf wetness duration affected the length of the incubation period (the time from inoculation to the appearance of the first lesions) only at sub-optimal temperatures. In Poland, Toscano-Underwood *et al.* (2001) found that the greatest numbers of leaf lesions were produced following 48 h of leaf wetness at 15-20°C for both L. maculans and L. biglobosa ascospores. As temperature decreased below 20°C, the length of the incubation period of the two groups of isolates increased. In Australia, Sosnowski *et al.* (2003) showed that leaves inoculated by pycnidiospores produced lesions from 6 to 25 days after inoculation as temperature decreased from 23 to 8°C. The variations among these studies suggest that factors such as inoculum used (ascospore or pycnidiospore) and pathogen genotypes may influence infection and disease development. There is a need to investigate the influence of environmental factors on incubation period on canola plants inoculated with ascospores in Australia. Additionally, the incubation period may differ between cultivars and between leaves at different positions (Poisson & Pérès, 1999b). This project aims to determine the effects of temperature and wetness duration on the incubation period of plants inoculated by the ascospores of *L. maculans* in a controlled environment.

1.4 Disease control

1.4.1 Disease resistance

Breeding for resistant cultivars is the most common and effective control of blackleg (Delourme *et al.*, 2006). Necrosis of guard cells adjacent to the infection site, production of phytoalexins, callose and lignin, and pathogenesis-related proteins are defence responses in canola to infection by *L. maculans* (Howlett, 2004). Breeding for resistance, at both seedling and adult plant stages, to *L. maculans* is a key requirement for sustainable management of blackleg (West *et al.*, 2001). In Australia, there has been extensive study to detect new sources of improved seedling and adult plant resistance to *L. maculans* (Marcroft *et al.*, 1999). Following the severe epidemics of the early 1970s, cultivars resistant to *L. maculans* were produced that allowed the re-establishment of the canola industry (Salisbury *et al.*, 1995). However, the enhancement of resistant cultivars has led some growers to shorten rotations and increase minimal tillage, which has increased inoculum pressure and resulted in severe epidemics. On the other hand, genetic recombination of *L. maculans* on residue may produce pathotypes capable of infecting important sources of resistance (Gladders *et al.*, 2006; Petrie, 1995b); such pathotypes have already been found in Australia (Ballinger *et al.*, 1991; Gladders *et al.*, 2006). Use of new cultivars with major gene resistance without a background of quantitative resistance may have contributed to the breakdown of resistance to blackleg in Australia (Sprague *et al.*, 2006). Several Australian cultivars, Dunkeld, Monty, Karoo and Ripper, are no longer grown due to susceptibility to blackleg. General strategies, such as rotation of various resistance genes in space and time and use of mixtures of cultivars with different resistance genes, could improve the durability of resistance genes (Gladders *et al.*, 2006).

Liu *et al.* (2006) found evidence of induced resistance in canola against *L. maculans*. Pre-treatment of canola leaves with ascospores of *L. biglobosa* and chemical defence activators, acibenzolar-S-methyl or menadione sodium bisulphate, increased incubation time and decreased blackleg severity on plants inoculated with *L. maculans* (Liu *et al.*, 2006). This research may, in time, contribute to new strategies for managing blackleg.

1.4.2 Stubble management

In all regions, stubble management and crop rotation reduce the risk of blackleg (West *et al.*, 2001). Currently, destruction of blackleg-infested stubbles by raking, burying or burning is recommended and improved methods for stubble management and increased decomposition are being sought (Barbetti & Khangura, 2000). In China and India, intensive cultural practices such as removal of the whole plant at harvest and subsequent flooding of canola fields for the following rice crop result in the destruction of inoculum. In Europe, deep ploughing to bury stubble, followed by minimal tillage or direct seeding to prevent the stubble from being brought back to the soil surface, is recommended but is difficult on heavy soils (Gladders & Musa, 1980). Burying blackleg-infested canola stubble not only speeds up the rate of break-down but also prevents ascospores from being discharged into the air (Kharbanda & Ostashewski, 1997; Turkington *et al.*, 2000b; Turkington *et al.*, 2000a). The weight reduction of blackleg-infected residues was more rapid in stubble buried in soil (40%) than unburied stubble (27%). However, Blenis *et al.* (1999) showed that mean weight reduction, for stem pieces of canola cultivars, ranged from 35 to 55-60% in 1994-1995 and from 35-40 to 45-50% in 1995-1996 for surface-placed and soil-incorporated stubbles, respectively, within a 10-12 month period. It was

suggested that differences in environment and soil conditions might lead to the variation between these two observations (Turkington *et al.*, 2000b).

Although burning of infested canola stubble is an effective practice to decrease inoculum levels in Australia (Bokor *et al.*, 1975) and England (Gladders, 1978), it results in air pollution and loss of organic matter. Fertilization of soil with N fertilizers, no-plough and inversion cultivations had no effect on intensity of leaf infection on canola in Germany, however, the severity of stem infections was slightly higher after minimum tillage than after ploughing (Söchting & Verreet, 2004). Sanitation is another cultural strategy to avoid carrying over the pathogen inoculum on blackleg-infested residues by farm machinery.

1.4.3 Crop rotation

Crop rotation is one of the most effective cultural practices available to producers to control blackleg of canola. Because ascospores can be released from infested residues left in or adjacent to fields for as long as the residues persist, isolation of the crop from stubble of the previous season is critical to minimise infection. Therefore, in south-eastern Australia, 4-year or shorter rotations are considered to be effective if canola crops are sown at least 200 m from canola stubble from the previous season, with 400 m being preferable (Marcroft *et al.*, 2003b). Controlling wild mustard and volunteer canola on summer fallow and in cereal crops, as the sources of inoculum, is also important in the rotation (Petrie, 1979).

The length of time for crop rotation depends on stubble persisting between canola crops and the potential of *L. maculans* to survive in and release ascospores from residue over time (Gabrielson,

1983). Therefore, understanding this aspect of blackleg epidemiology for each environment is critical to developing successful crop rotation programs involving canola.

1.4.4 Disease escape

Delayed sowing of the canola crop as a means of blackleg control in Australia (McGee, 1977). The decline of blackleg severity in canola crops sown later in the year is likely to be due to fewer ascospores being present later in the year. Similarly, in England, growers sow winter canola early so that the plants are well established before maximal ascospore discharge (Gladders & Musa, 1980). In contrast, in Western Australia, no correlation was observed between sowing date and disease severity or incidence (Khangura & Barbetti, 2001). The variation between these findings is likely to be due to the differing climatic conditions, which influence the timing of ascospore discharge and infection.

1.4.5 Chemical control

Current fungicides are effective as protectants for a limited period due to degradation, leaf expansion and the production of new untreated leaves (West *et al.*, 2000). Foliar spraying with triazole compounds such as hexaconazole, triadimefon, diconazole and uniconazole has been found effective to control blackleg (Rempel & Hall, 1993). Flutriafol, coated on fertiliser and applied as an in-furrow treatment, greatly reduce the disease severity (Ballinger *et al.*, 1988a; Ballinger *et al.*, 1988b). Although leaf infection was reduced by tebuconazole application in autumn as well as in spring, control of blackleg was only observed after the autumn treatment in Germany (Kruse & Verreet, 2005).

The value of different combinations of fungicide seed treatments or foliar fungicide sprays to control blackleg in different areas depends upon the economics of the crop and the epidemiology of blackleg. In Australia, using foliar fungicides on canola cultivars with little or no resistance proved to be ineffective to control the disease (Brown *et al.*, 1976). Fungicide use is economic and effective only when applications are correctly timed, so there is a need for a forecasting scheme to predict the severity of epidemics at the time when chemical control could be effective (Gladders *et al.*, 1998).

1.4.6 Biological control

Two species of "bird nest" fungus, *Cyathus striatus and C. olla,* have been suggested as biological control agents of *L. maculans.* These fungi reduce the residue food base on which *L. maculans* survives between canola crops. Therefore, they are able to decrease the level of inoculum produced on stubble to initiate infection (Tewari *et al.*, 1997). Furthermore, *C. striatus* produces cellulase and lignase and has a greater ability for primary resource capture than *L. maculans* (Maksymiak & Hall, 2000). *Paenibacillus polymyxa* PKB1 has also been found to produce two antifungal peptides, which reduce the growth of *L. maculans* in culture medium, on leaves, on stems and on stubble (Karbanda *et al.* 1999). This bacterium has potential for use as a direct biological fungicide, because experiments have revealed that application of several fungicides and herbicides for use on canola did not affect *P. polymyxa.*

1.5 Forecasting of disease

Precise forecasting of blackleg could improve disease control and reduce fungicide applications (West *et al.*, 1999a). The accuracy of forecasting models could be improved by including parameters relating to the maturation of ascospores in pseudothecia (see section 1.3.2.2), the release of ascospores (see section 1.3.2.3) and the appropriate conditions for the appearance of infection (see section 1.3.3), as they affect the onset, intensity and duration of leaf infection in the field. West *et al.* (1999b) optimised the use of fungicides to control blackleg in the UK and evaluated the relationship between pseudothecial maturation, spore release and disease incidence as part of a disease forecasting scheme. They suggested that monitoring pseudothecium maturation, timing of spore release and favourable infection conditions could be used to optimise fungicide applications. Whereas models based on the occurrence of mature pseudothecia capable of releasing ascospores have been developed in France (Bernard *et al.*, 1999) and Western Australia (Salam *et al.*, 2003a; Salam *et al.*, 2003b), as described in section 1.3.2.2, such models have not yet been developed for South Australia. Likewise, disease survey data, weather and crop resistance were used to develop a statistical model to predict the incidence (percentage of plants affected) of light leaf spot caused by *Pyrenopeziza brassicae* on canola in England and Wales (Welham *et al.*, 2004).

This project will determine the influence of temperature and wetness duration on pseudothecium maturity, ascospore release and incubation period of *L. maculans* in a controlled environment to provide further information towards developing an accurate forecasting model for future use in South Australia. It will also extend our knowledge on the survival of the fungus in different soil types and the effect of temperature on ascospore germination.

1.6 Conclusion

Blackleg is an important disease of canola. Understanding the epidemiology of the disease helps to manage it effectively. In order to develop a forecasting model to predict blackleg, information on the influence of environment on different stages of disease development is required. It is important to gain more information on the effects of temperature and wetness duration on pseudothecium maturation, ascospore discharge and incubation period. Such information is needed to predict the onset of blackleg in the field and there are a number of forecasting models developed on the basis of the above factors. However, results obtained from other countries or other regions of Australia, with different environmental conditions and *L. maculans* genotypes, may not be suitable to develop an accurate forecasting model for use in south-eastern Australia. The effect of temperature on the germination of ascospores also needs to be examined, using Australian genotypes of *L. maculans*.

Further information on survival of *L. maculans* is required. For example, understanding the influence of soil type and microbial activity on the survival of the fungus on stubble will provide useful information for developing improved management strategies.

1.7 Research objectives

The objectives of this research were to:

- Investigate the influence of temperature, wetness and darkness on *L. maculans* pseudothecium formation on stubble, and study the effect of temperature on ascospore discharge.
- Study the effect of temperature and cultivar on germination and hyphal growth from ascospores of *L. maculans* on canola leaf and cotyledon.
- Examine the effect of temperature, cultivar and leaf position on incubation period (time from inoculation to appearance of first lesions) of *L. maculans* on canola.
- Determine the effect of burial of stubble in two soil types on pathogen survival, pathogenicity, associated mycobiota on the stubble and changes in soil microbiota.
- Study the interactions between *L. maculans* and mycobiota from the stubble and soil.

Chapter 2. General material and methods

2.1 Plant materials

2.1.1 Canola cultivars

Canola cultivars with different blackleg resistance ratings were used for experiments described in this thesis (Table 2.1). Canola seed was supplied by Mr Trent Potter, South Australian Research and Development Institute (SARDI), Struan, South Australia. Blackleg ratings and status of the cultivars were obtained from the blackleg resistance rating system published by the Canola Association of Australia, from nursery trials around Australia by Departments of Agriculture and private breeding companies (Potter & Stanley, 2002).

Table 2.1. Blackleg ratings and status of canola cultivars used (Potter & Stanley, 2002).

Cultivar	Maturity	Blackleg rating[1]	Blackleg status
Hyola 60[2]	Mid	9	Resistant
Ripper	Mid	6.5	Resistant
ATR-Beacon	Mid	6	Resistant
Monty	Early	4.5	Moderately resistant
Karoo	Early-mid	3.5	Moderately susceptible
Q2[3]	Mid-late	2	Susceptible

[1] 1 is most susceptible and 9 is most resistant.

[2] In some regions of Australia, Hyola 60 is no longer resistant to blackleg (Sosnowski *et al.*, 2004; Sprague *et al.*, 2006)

[3] Q2 is a Canadian cultivar which is highly susceptible to blackleg in Australia.

2.1.2 Plant growth

2.1.2.1 Potting soil

UC soil (Baker, 1957) was used in all glasshouse or growth cabinet experiments and was produced by Plant Growth Services, Plant Research Centre, Waite Campus. Waikerie sand, 1200 l (2 bins), was steam sterilized at 100°C for at least 45 minutes. Peatmoss, 750 l (3 bales), was added and mixed for 5 minutes. Extra water was added at this stage, if required. After 20 minutes 1.4 kg calcium hydroxide (hydrated lime) and 2 kg calcium carbonate were mixed in for 5 minutes. These three bins of UC base were left for at least 24 h to cool. Each bin of UC base was put into a mixer and 1.5 kg of Osmocote Mini® fertilizer (Scotts Australia Pty Ltd, New South Wales, Australia) was added and mixed for 5 minutes.

2.1.2.2 Plant growth stage

A code for stages of development in canola revised by Sylvester-Bradley (1985), described in appendix 1, was used to describe plant growth stage (GS). Canola seedlings used in the experiments were at early leaf production stage (GS 0-1.4); leaves were counted if they were separated from the terminal leaf cluster of the canola plant (Sylvester-Bradley, 1985).

2.1.2.3 Greenhouse conditions

Plants were placed on benches and exposed to natural daylight. Pots were watered by hand once a day with tap water. The average temperature in the greenhouse was maintained at 18-29°C using an evaporative cooling and convection heating system.

2.1.3 Canola stubble

Canola stubble used in experiments was supplied by Mr Trent Potter, SARDI, Struan, South Australia. The blackleg-affected stubble, which consisted of 40-80 cm long stem and root pieces, was collected in June 2003 (harvested in January 2003) and January 2004 (immediately after harvest) from a blackleg disease nursery, containing 100-150 commercial cultivars or breeding lines. The blackleg-resistance rating of cultivars and lines in the nursery ranged from 1 to 9, with most in the 5-7 range. About 5% of the cultivars and lines evaluated in the disease nursery had resistance derived from *Brassica rapa* subsp. *sylvestris* while the rest of the lines had polygenic resistance. The stubble was dried immediately on arrival at the laboratory, using an oven at 30°C for 72 h and then stored in polypropylene bags at room temperature (approximately 22°C) until use.

2.2 Controlled environments

2.2.1 Controlled environment cabinet

An Environ Air controlled environment chamber (100 x 120 x 145 cm) was used. Artificial lighting was provided using two cool white 115W fluorescent tubes (VHO Sylvania, F48T12) which were controlled by a Therben (WF 64CA7) timer set for a 12 h photoperiod. Temperature settings were kept constant by an RKC REX-C100 electronic temperature controller. The temperature inside the cabinet was monitored weekly using a Hastings Data Logger (Gemini Data Loggers (UK) Ltd).

2.2.2 Incubators

Two incubators (Glyson Germination Incubator, Crown Scientific Pty Ltd, New Zealand) were used in experiments at designated temperatures under 12 h light (fluorescent NEC T8 FL20SSBR/18-HG, Japan; black light NEC T5 8W/FL8BL, Japan) followed by 12 h darkness. The accuracy of the temperature inside the incubators was monitored weekly using Hastings Data Loggers (Gemini Data Loggers (UK) Ltd, UK).

2.3 Fungi

2.3.1 *L. maculans* on canola stubble

L. maculans was isolated from blackleg cankers on the stubble described in section 2.1.3 and identified based on the characteristics described by Punithalingam & Holliday (1972). Ten stem pieces (1-cm length) were cut from randomly selected stubble, rinsed with tap water to remove soil, and left to dry at room temperature. Stems were surface sterilized in sodium hypochlorite solution (0.5%) for 3 min, rinsed in sterile distilled water and left on sterile filter paper for 2 h to dry in a laminar flow cabinet. The samples were plated onto potato dextrose agar (PDA, described in appendix 2) and incubated at 20 ± 2°C under fluorescent light and darkness for 12 h each (see section 2.2.2) for at least 2 weeks. Colonies of *L. maculans* were subcultured by transfer of hyphal tips to fresh PDA until pure.

2.3.2 Isolation and purification of fungi from canola stubble

In certain experiments, fungal species were isolated from canola stubble on dilute V-8 juice agar (DV8A, described in appendix 2). Single spore-derived cultures of fungi isolated from the stubble in experiments were prepared by placing a 1 mm^2 plug of spore-bearing mycelium on PDA in 5 ml of sterile RO water. A spore suspension was obtained by gently shaking and stirring the plug in water. An aliquot, 1 ml, of the suspension was spread evenly over thin water agar (WA, described in appendix 2) and incubated on a bench under fluorescent (Phillips TDL 36W/840, Thailand) and NUV (NEC T10 40W/FL40SBL, Japan) light at room temperature (approximately 22°C). Germinating single spores were located using a light microscope (Olympus BH-2, Japan) at 100 × magnification after 24 h of incubation. Single germinated spores were transferred to PDA plates (9 cm diameter). The cultures were incubated at room temperature as above for at least 2 weeks to produce spores. For non-sporulating fungi, colonies on PDA were subcultured with hyphal tips until pure.

2.3.3 Storage of fungal isolates

Fungal isolates were cultured on PDA in Petri dishes and slant tubes, as described in appendix 2, and stored at 5°C. Plugs (3 mm diameter) of pure cultures on PDA were removed using a cork borer and transferred to sterile RO water for long-term storage at 5°C.

2.4 Statistical analysis

Data from experiments were subjected to analysis of variance (ANOVA) and regression analysis using GenStat version 6.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, 2002) or GenStat version 8.2 (Lawes Agricultural Trust, Rothamsted Experimental Station, 2005).

Chapter 3. Formation of pseudothecia and ascospores of *L. maculans*

3.1 Introduction

Inoculum of *L. maculans* develops on infested canola stubble left in the field after harvest. In the field, pseudothecia of *L. maculans* appear on the residues of canola from a week (Hershman & Perkins, 1995) to 10 months (Petrie, 1986) after harvest and continue to form as long as stubble persists in soil. Ascospore discharge occurs after wetting of stubble by rain (Guo & Fernando, 2005; Huang *et al.*, 2005; Pérès & Poisson, 1997) and dew (Kruger & Wittern, 1985; McGee, 1977). Air temperature also is reported to affect ascospore discharge (Guo & Fernando, 2005; Kruse & Verreet, 2005; McGee, 1977; Pérès *et al.*, 1999). In Australia, harvest is generally in spring, November-December (Sosnowski, 2002) and ascospore release begins in May in response to winter rainfall, which also stimulates the emergence of canola seedlings (McGee, 1977).

In general, climatic and biological observations in the field have shown that the beginning of ascospore discharge depends mainly on weather conditions (Gabrielson, 1983; McGee, 1977; Pérès & Poisson, 1997). Petrie (1994) showed that the optimum temperature for ascospore production by *L. maculans* was 15°C and peak sporulation occurred at 20°C in Canada. In western Canada, ascospore dispersal peaked several hours after rainfall of ≥ 2 mm and continued for approximately 3 days after such events (Guo & Fernando, 2005). McGee (1977) determined patterns of ascospore discharge in Victoria, Australia, from May to October using a Burkard spore trap, continuously sampling air at the rate of 10 l per minute onto slides coated with ovalbumin. The peak of ascospore capture often occurred in June, July and August when temperature is 8-12°C and rainfall >1.0 mm (McGee, 1977). Karbanda and Ostashewski (1997) suggested that differences in the timing of pseudothecium formation and ascospore discharge

from stubble between regions are due to the influence of environmental factors on the differential development of pseudothecia.

In France, a mean temperature of 14°C and a high frequency of rainfall, 2.5 mm every 3-4 days in the field, or 100% relative humidity in a controlled environment, were the most suitable conditions for the appearance of pseudothecia on canola stubble (Pérès *et al.*, 1999). At 10°C, mature pseudothecia were not observed, whereas at 14, 16, 18 and 20°C pseudothecia matured after 14, 17, 21 and 34 days, respectively, in 95-100% relative humidity in a controlled environment (Pérès *et al.*, 1999). The time taken for pseudothecia of *L. maculans* and *L. biglobosa* to mature on naturally infested stubble decreased nearly linearly when temperature increased from 5 to 20°C under continuous wetness in a controlled environment but was longer in natural conditions, particularly in dry weather in the UK (Toscano-Underwood *et al.*, 2003). The first capture of ascospores and, consequently, first Phoma leaf spots, was usually observed after rainfall and in decreasing temperature at the end of summer in France (Pérès *et al.*, 1999). In south-eastern Australia, significantly more ascospores were released from pseudothecia on 6-month-old stubble in a high rainfall region than stubble of the same age in a medium rainfall region, which in turn released more than stubble in a low rainfall region (Marcroft *et al.*, 2003a). Additionally, Marcroft *et al.* (2003a) reported that in all regions, fields with 6-month-old stubble released 30 times more ascospores than fields with older stubble (18-42 months). In Western Australia, pseudothecia matured at different times in areas with different rainfall and maturation was considered likely to be affected by temperature and rainfall. In addition, the seasonal pattern of ascospore discharge (Khangura *et al.*, 2001) was different in different years of the study or different regions. At Mt Barker, Western Australia, ascospore release from stubble of the previous year's crop started in March 2000 and peaked in May, whereas it peaked in June in

1999. At East Chapman, ascospore discharge started in July, peaked in August and declined in September 2000 (Khangura *et al.*, 2001). Therefore, pseudothecium maturation and ascospore discharge occurred earlier in regions with higher rainfall in Western Australia. Although forecasting models have been developed to predict the onset of maturity of pseudothecia in France (Bernard *et al.*, 1999) and Western Australia (Salam *et al.*, 2003a; Salam *et al.*, 2003b), collection of climatic and biological data is required in South Australia to develop a forecasting model for this state. Although the potential of host resistance to reduce production of pseudothecia and ascospores on stubble of canola was demonstrated (Marcroft *et al.*, 2004), effects of other factors such as interrupted wetness and darkness on inoculum production on stubble are little understood.

A four-stage scale has been used previously to examine the development of pseudothecia of *L. maculans*, however, only differentiation of ascospores at C and D stages was described (Bernard *et al.*, 1999), whereas the differentiation of pseudothecia and asci was not included. Toscano-Underwood *et al.* (2003) modified the classification of Bernard *et al.* (1999) and included descriptions for asci and ascospores at all stages of maturity (A, B, C, D & E). However, this modified scale requires the measurement of the length of asci (for stages B, C & D) and ascospores (for stage D) to group pseudothecia. The use of the latter scale for studies that examine the differentiation stage of a large number of pseudothecia is difficult and time-consuming. Therefore, in this study, a modified "easy to use" scale was developed for assessing large numbers of pseudothecia quickly.

In South Australia, the effect of environmental factors on pseudothecium maturation on stubble and also the timing of this phenomenon in the field are little understood. In order to predict the

timing of inoculum production in the field, identification of appropriate temperature and wetness periods for pseudothecia to form and mature is required. In this regard, McGee (1977) showed that laboratory-induced ascospore discharge from stubble closely approximates natural discharge. Therefore, this study examined the influence of temperature, darkness, continuous and discontinuous wetness on pseudothecial maturation on stubble, collected from South Australian fields of canola, in a controlled environment. The effect of temperature on ascospore discharge from pseudothecia on stubble was also studied.

3.2 Materials and Methods

The scale of Bernard *et al.* (1999) was modified to allow easy classification of *L. maculans* pseudothecia based on microscopic observation (Table 3.1).

Table 3.1. Pseudothecium maturation stages of *Leptosphaeria maculans* (modified from the scale developed by Bernard *et al.* (1999)).

Stage[1]	Pseudothecia	Asci	Ascospore
P	Absent	Absent	Absent
A	Not mature	Not mature	Absent
B	Not mature	< 8 spores	< 5 septa
C	Not mature	8 spores	5 septa
D	Mature	Mature	Mature[2]
E	Empty	Empty	Discharged

[1] A pseudothecium was considered to have reached each stage of maturation when the first observed asc and ascospore in the pseudothecium had reached the relevant stage of development.
[2] Ascospore is 5-septate, constricted at first septum and yellow (Shoemaker & Brun, 2001).

3.2.1 Effect of temperature on pseudothecium formation under continuous wetness

Pieces of tap root and stem base (*ca* 20-30 cm in length) of one-year-old canola stubble with blackleg cankers (without pseudothecia) were obtained from a blackleg disease nursery in January 2004 (see section 2.1.3). Stubble pieces were soaked in sterile distilled water for 12 h. Plastic trays (45 × 31 × 5 cm) were lined with sterile sand (1 cm depth), which had been thoroughly moistened with distilled water. Ten replicate stubble pieces were placed in a tray for each temperature treatment. Each tray was placed in a plastic bag that had been sprayed inside with distilled water to maintain 100% relative humidity. Trays were then placed in the two incubators adjusted, sequentially, to 5, 10, 15 or 20°C with a 12 h photoperiod (see section 2.2.2). The experiment was conducted three times.

If pseudothecia at any stage of differentiation were present, five pseudothecia were cut randomly from each of the 10 stubble pieces and placed, separately, in a drop of water on a glass slide. Gentle pressure was applied to force asci from the pseudothecia. Then each pseudothecium was grouped into one of five maturity classes (A, B, C, D or E), using the scale shown in Table 3.1. Development of pseudothecia and the number of pseudothecia in each stage of maturity were assessed every 5 days until most pseudothecia had matured. At the last assessment, the density of pseudothecia on each piece was assessed as follows. A segment of 0.5 × 1 cm was cut from each piece and the number of pseudothecia was counted using a dissecting microscope at 25 × magnification. The average density of pseudothecia in each tray was calculated as the mean density on the 10 stem pieces.

3.2.2 Effect of darkness on pseudothecium formation under continuous wetness

Ten replicate stubble pieces were prepared as described in section 3.2.1 and were then placed on sand in one tray. The tray was placed in a plastic bag, sprayed inside with distilled water to maintain 100% relative humidity, and then covered with foil to exclude light. The tray was incubated (see section 2.2.2) at 15°C in darkness, so that stubble pieces received no light except when they were assessed for the development of pseudothecia. The experiment was conducted three times. Development of pseudothecia on stubble pieces, the number of pseudothecia at each stage of maturity and the density of pseudothecia on each piece were assessed, as in section 3.2.1, until most pseudothecia had matured.

3.2.3 Effect of discontinuous wetness on pseudothecium formation

Stubble pieces were prepared as described in section 3.2.1 and ten replicate pieces were then placed on sand in each tray. Three trays (not placed in plastic bags) were placed in the incubator adjusted to 15°C with a 12 h photoperiod (see section 2.2.2). One of three trays was sprayed with distilled water from a hand-held sprayer, to produce a thin film of water on the stubble, once a day (at 9 am), the other one twice (at 9 am and 5 pm), and the last one three times a day (at 9 am, 1 and 5 pm), every day until the end of the experiment. The experiment was conducted three times. Development of pseudothecia on stubble pieces and the number of pseudothecia at each maturity stage were assessed every 5 days until most pseudothecia had matured, as described in section 3.2.1. The density of pseudothecia on each piece was assessed at the end of each repetition of the experiment.

3.2.4 Development of a method to study the effect of temperature on ascospore release in a controlled environment

Stubble incubated at 15°C in the experiment described in section 3.2.1 was air-dried for 24-48 h and stored in polypropylene bags at room temperature (approximately 22°C) until use.

3.2.4.1 Assessment of ascospore release inside a plastic tray

Twenty replicate pieces of stubble, *ca* 10 cm length, with mature pseudothecia were cut into 3-4 segments, and then pieces were soaked in sterile distilled water for 1-2 minutes. Stubble segments were placed in a plastic tray (45 × 31 × 5 cm) lined with regular Chux cloth wipes (60 × 30 cm, Clorox Pty Ltd, Australia), which had been thoroughly moistened with distilled water. Four glass slides, on which a uniform, thin layer of Vaseline petroleum jelly was spread in order to catch ascospores released from stubble, were placed among the stubble in a vertical position with the aid of small pieces of white polystyrene packaging material. Then each tray was placed in a plastic bag, sprayed inside with distilled water to maintain 100% relative humidity. Trays were then placed in the incubator adjusted to 20°C with a 12 h photoperiod (see section 2.2.2) for 24-48 h. The experiment was conducted three times.

The slides were assessed at 4-hourly intervals using a light microscope at 100 × magnification to count the number of ascospores and also to distinguish between ascospores of *L. maculans* and spores from other fungi present on the stubble. Slides were then replaced with fresh Vaseline-coated slides.

3.2.4.2 Assessment of ascospore release inside a Petri dish

Six stubble pieces (3 cm length, with mature pseudothecia) were attached to the underside of a Petri dish lid using petroleum jelly. Stubble pieces were then flooded with sterile distilled water for 15 s, the water then poured off and the lid placed over the base. Nine replicate Petri dishes were prepared for each temperature treatment (5, 10, 15 & 20°C) and incubated at the designated temperature.

Ascospore discharge from pseudothecia was assessed at 2-hourly intervals, as follows, until no more ascospores were detected. After each 2 h of incubation at each temperature, the bases of the nine Petri dishes were replaced with nine fresh bases, so that the lids of the nine replicate Petri dishes, containing stubble pieces, remained inside the incubator until the end of experiment. Then 1 ml sterile distilled water was added to each Petri dish base, and the ascospores that had been released were suspended using an artist's paint brush. Three replicate aliquots, 25 μl each, of the suspension of ascospores in each Petri dish base were placed on glass slides. After placing a cover slip on the drop of ascospore suspension on each slide, the number of ascospores in 25 μl was counted using a light microscope at 100 × magnification. In addition, the duration of ascospore discharge at each temperature was recorded.

3.2.5 Statistical analysis

The incubation time (days) required to reach each stage of differentiation and maturity of pseudothecia was the day when the number of pseudothecia at each stage of maturity reached its maximum over the assessments at each temperature. Data for the time to reach each stage of maturity and data for the density of pseudothecia at different temperatures from three repetitions

of the experiment were subjected to ANOVA using GenStat version 6.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, 2002). ANOVA was used to examine differences between the time taken to reach each stage of maturity and the density of pseudothecia on stubble incubated in a 12 h photoperiod, and those in darkness under continuous wetness at 15°C. ANOVA was also used to analyze the data for the time taken to reach each stage of maturity and the density of pseudothecia under discontinuous wetness (1, 2 and 3 sprays of water per day), also to compare the time taken to reach each stage of maturity and the density of pseudothecia incubated under continuous wetness and those under discontinuous wetness (K. Dowling, pers. com., 2005). Data for the time taken for pseudothecia to mature at different temperatures from three repetitions of the experiment under continuous wetness in a 12 h photoperiod were used to develop a power regression curve.

The data for the number of ascospores released into Petri dishes at the temperatures tested were $\log_e$-transformed before analysis to improve the homogeneity of the data. The effects of the temperatures tested on ascospore discharge were analyzed by linear mixed models (REML) using GenStat version 8.2 (Lawes Agricultural Trust, Rothamsted Experimental Station, 2005). REML was used due to the unbalanced experimental structure (unequal number of sampling times at different temperatures), and Wald tests (GenStat version 8.2, Lawes Agricultural Trust, Rothamsted Experimental Station, 2005) were used to examine temperature and time effects. Mean values for $\log_e$-transformed data were calculated and back-transformed.

3.3 Results

3.3.1 Effect of temperature on pseudothecium formation under continuous wetness

Pseudothecia of *L. maculans* differentiated and matured at 5-20°C under continuous wetness and a 12 h photoperiod in a controlled environment. Temperature significantly affected (LSD = 6.8; $P < 0.05$) the mean incubation time required for pseudothecia to reach each stage of maturity (Figure 3.1). The mean time required for pseudothecia to reach each stage of maturity was longer ($P < 0.05$) at lower than higher temperatures over 5 to 15°C and was similar ($P > 0.05$) at 15 and 20°C. The mean time required for immature pseudothecia to appear on stubble (stage A) decreased ($P < 0.05$) from 29.3 days at 5°C to 8.5 days at 15°C and was similar between 15 and 20°C. The incubation time for pseudothecia to reach stage D significantly ($P < 0.05$) decreased from 58.3 days at 5°C to 22.2 days at 15°C and was 25.7 days at 20°C. The polynomial regression curve of mean incubation time ($R^2 = 0.97$) until the number of pseudothecia was maximal at stage D is shown in Figure 3.2. The mean time required for pseudothecia to release ascospores (stage E) decreased ($P < 0.05$) from 64 days at 5°C to 28.5 days at 15°C and was 31.7 days at 20°C (Figure 3.1). At each stage of maturity, the difference in the incubation time was more marked when comparing 5 with 10°C than 10 with 15°C or 10 with 20°C.

At 5°C, the mean incubation time required for transition from stage A to B and stage B to C significantly ($P < 0.05$) increased from 29.3 to 45.3 days and from 45.3 to 54 days at stage C, respectively, and was similar over C-E. At 10-20°C, the mean incubation time required for transition from each stage of maturity to the next stage was similar, except for transition from

stage B (reached at 24.7 days) to stage C (reached at 35.7 days) at 10°C and from stage A (8.7 days) to stage B (15.7 days) at 20°C.

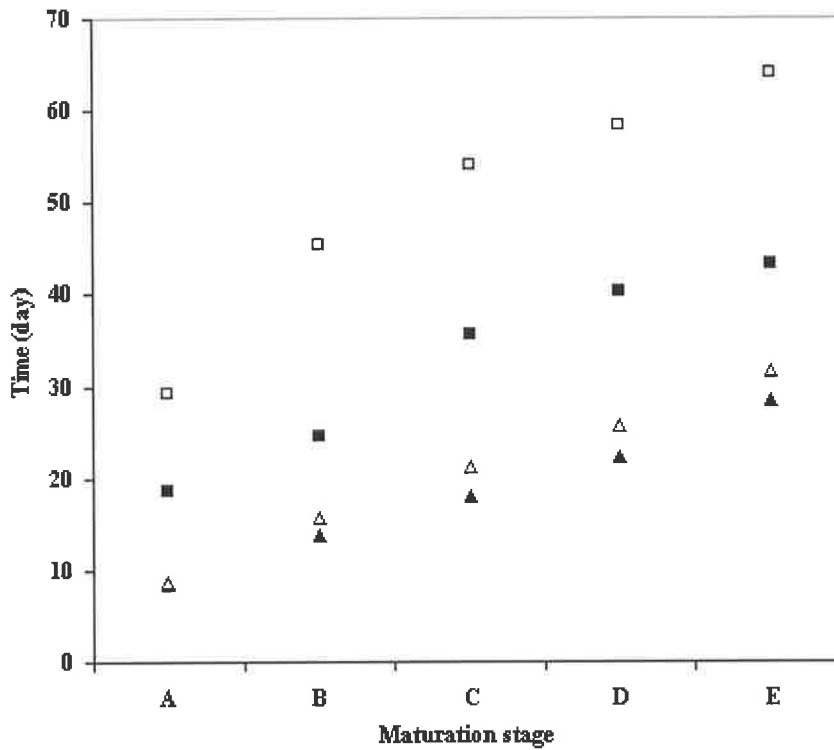


Figure 3.1. Time required for pseudothecia of *Leptosphaeria maculans* to reach each stage of maturity (such that the number of pseudothecia at that stage was maximal) at 5 (□), 10 (■), 15 (▲) and 20°C (△) in a controlled environment; LSD = 6.8; P < 0.05.

Pseudothecial development proceeded through stages A, B, C, D and E under continuous wetness at 5-20°C (Figure 3.3). The average maximum number of pseudothecia at stages A, B and C observed in the three repetitions of the experiment was greater at 5°C than at 10-20°C. More pseudothecia at stages D and E were observed at 15°C than at the other temperatures tested. The rates of maturation of pseudothecia (i.e. time taken to reach stage D) were similar at 5 and 10°C,

and were lower than those at 15-20°C (Figure 3.4). Maturation rate was greatest at 15°C in comparison with the other temperatures tested.

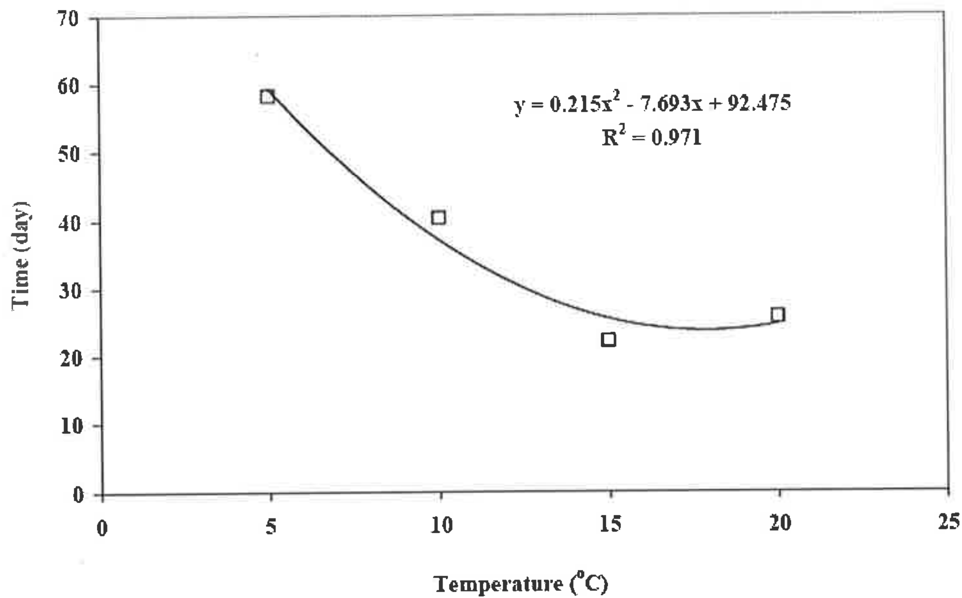


Figure 3.2. Relationship between temperature and maturation of pseudothecia of *Leptosphaeria maculans* at 5-20°C in a controlled environment; the time taken for pseudothecia to mature was the day when the number of pseudothecia at D stage was maximal over the assessments at each temperature.

Temperature had little effect on the density of pseudothecia of *L. maculans* that developed on canola stubble in the controlled environment (Table 3.3) and only the mean density of pseudothecia at 15°C was significantly greater (LSD = 9.1; $P < 0.05$) than that at 20°C.

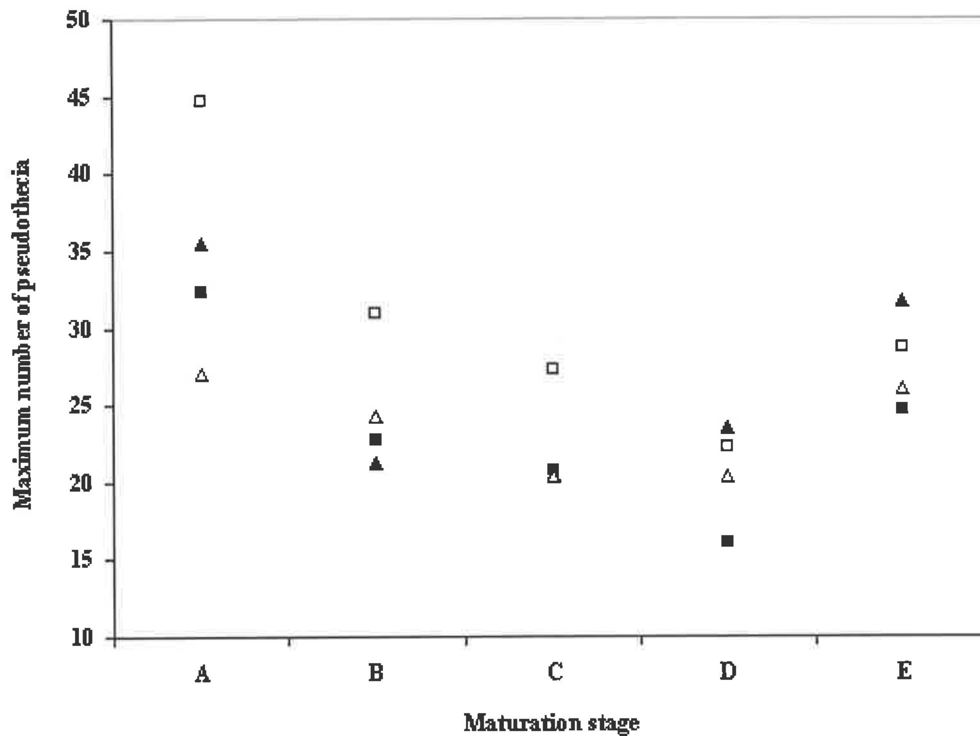


Figure 3.3. Average maximum number of pseudothecia observed at each stage of maturity from three repetitions of the experiment at 5 (□), 10 (■), 15 (▲) and 20°C (△) in a controlled environment.

3.3.2 Effect of darkness on pseudothecium formation under continuous wetness

Pseudothecia of *L. maculans* differentiated, matured and released ascospores at 15°C in darkness and continuous wetness. There was no significant difference in the mean time required for pseudothecia to differentiate and mature at 15°C in the 12 h photoperiod and in darkness, except for the time to reach stage A (Table 3.2). In darkness, the mean time taken for pseudothecia to mature from one stage to the next stage increased (LSD = 3.1; $P < 0.05$), except for the transition from A to B. The mean density of pseudothecia formed in continuous darkness (17.5) was

significantly less (LSD = 9.3; $P < 0.05$) than that with a 12 h photoperiod (53.4) at 15°C (Table 3.3).

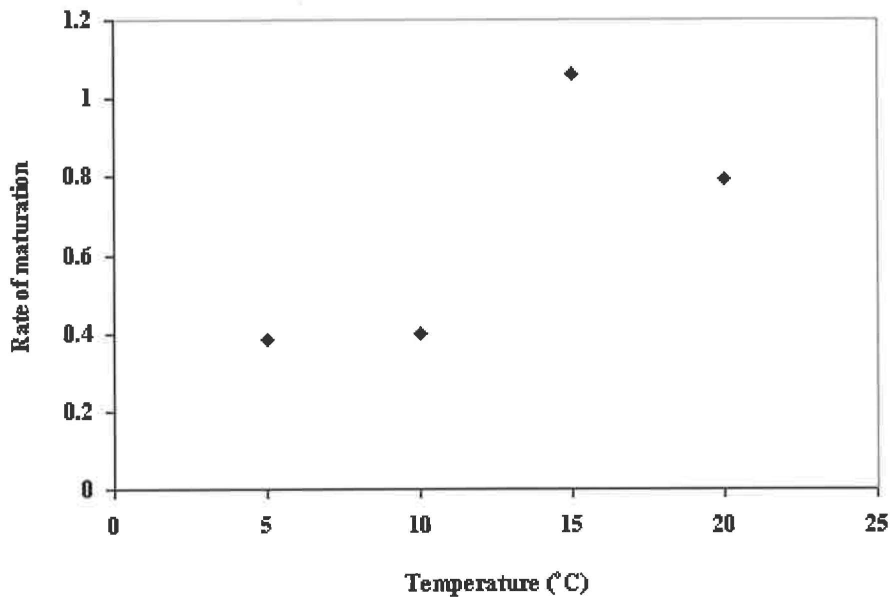


Figure 3.4. The rate of maturation of pseudothecia of *Leptosphaeria maculans* at 5-20°C in a controlled environment; rate of maturation was calculated as average maximum number of pseudothecia observed at stage D from three repetitions of the experiment at each temperature divided by average time (day) for pseudothecia to reach stage D.

3.3.3 Effect of discontinuous wetness on pseudothecium formation

Pseudothecia of *L. maculans* differentiated, matured and released ascospores at 15°C under discontinuous wetness (2-3 sprays of water per day) and a 12 h photoperiod in a controlled environment. Pseudothecia were not observed on stubble that received one spray of water per day (Table 3.2). Discontinuous wetness with two sprays per day increased (LSD = 5.3; $P < 0.05$) the

mean time taken for pseudothecia to mature (to reach stage D) to 53.3 days in comparison with 22.2 days in continuous wetness. Pseudothecia took longer to mature in discontinuous wetness with three sprays per day (46.7 days) than in continuous wetness. The time taken for pseudothecia to reach each stage of maturity (stages A to E) in discontinuous wetness (two sprays per day) was significantly longer ($P < 0.05$) than that in continuous wetness. Differentiation of pseudothecia on stubble receiving two sprays per day took significantly longer (LSD = 6.1; $P < 0.05$) than that with three sprays per day, although the time to release ascospores (stage E) was similar.

Table 3.2. The incubation time (the day when the number of pseudothecia at each stage of maturity reached its maximum) required for pseudothecia of *Leptosphaeria maculans* to reach each stage of maturity at 15°C in a controlled environment.

Conditions		Stage of maturity				
		A	B	C	D	E
Continuous wetness & 12 h photoperiod		8.5[1]	13.7	18	22.2	28.5
Continuous wetness & Darkness		13.3[2]	15	20	25	30
Discontinuous wetness	1 spray	0[3]	0	0	0	0
& 12 h photoperiod	2 sprays	26.7[4, 5]	36.7	46.7	53.3	56.7
	3 sprays	20	25	36.7	46.7	51.7

[1] LSD = 6.8 for continuous wetness and 12 h photoperiod.

[2] LSD = 3.1 for darkness and for comparing darkness with continuous wetness plus a 12 h photoperiod.

[3] No pseudothecia formed under discontinuous wetness with one spray per day.

[4] LSD = 5.3 for comparing continuous wetness with discontinuous wetness plus two and three sprays per day.

[5] LSD = 6.1 for discontinuous wetness.

The mean density of pseudothecia on canola stubble that received two or three sprays per day at 15°C was similar (Table 3.3), but was significantly less than that in continuous wetness at the same temperature.

Table 3.3. Effect of temperature (5-20°C), darkness and discontinuous wetness (1-3 sprays of water per day) on the density of pseudothecia of *Leptosphaeria maculans* formed on canola stubble in a controlled environment.

Parameter		Density of pseudothecia
Temperature in 12 h photoperiod & continuous wetness	5°C	47.7[1]
	10°C	49.4
	15°C	53.4
	20°C	42
Darkness & continuous wetness	15°C	17.5[2]
Discontinuous wetness & 12 h photoperiod	1 spray per day	0
	2 spays per day	22.8[3, 4]
	3 spays per day	25.2

[1] LSD = 9.1 at 5-20°C in 12 h photoperiod and continuous wetness; $P < 0.05$.

[2] LSD = 9.3 for comparing in darkness to a 12 h photoperiod at 15°C.

[3] LSD = 5.1 in discontinuous wetness.

[4] LSD = 8.4 in discontinuous wetness with 2 and 3 sprays of water per day and in continuous wetness at 15°C.

3.3.4 Development of a method to study the effect of temperature on ascospore release in a controlled environment

3.3.4.1 Assessment of ascospore release inside a plastic tray

No ascospores were captured on glass slides covered with petroleum jelly.

3.3.4.2 Assessment of ascospore release inside a Petri dish

Ascospores of *L. maculans* were collected from pseudothecia on canola stubble into Petri dishes at 5-20°C. Pseudothecia needed to be moistened to release ascospores, as no ascospore was discharged from mature pseudothecia on air-dried canola stubble. The interaction between temperature and time affected the number of ascospores released from stubble (Table 3.4). The mean number of ascospores released was greater (LSD = 0.34; $P < 0.05$) at 20°C than the other temperatures tested at each time. Ascospores were not discharged after 10 h of incubation at 5-10°C, whereas ascospores were discharged until 10 and 12 h at 15 and 20°C, respectively.

At 5°C, the mean number of ascospores collected decreased (LSD = 0.48; $P < 0.05$) over time. At 10°C, ascospore discharge did not differ over 2 to 4 h and then decreased ($P < 0.05$) at 6 and 8 h. At 15°C, ascospore release increased ($P < 0.05$) at 4 h and then decreased over 6-10 h of incubation. At 20°C, the mean number of ascospores increased ($P < 0.05$) from 2 to 4 h of incubation and then decreased from 6 to 8 h and from 10 to 12 h of incubation.

Table 3.4. The effects of temperature, 5-20°C, and incubation time (h) on ascospore discharge from pseudothecia of *Leptosphaeria maculans* on canola stubble in a controlled environment.

Temperature	Incubation time (h)					
(°C)	2	4	6	8	10	12
5	2.53[1]	2.1	1.51	0.96	0	0
10	2.35	2.34	1.62	1.18	0	0
15	2.2	2.43	2.15	1.86	1.56	0
20	2.81	3.23	3.31	2.94	2.85	1.91
5	11.58[2]	7.17	3.51	1.62		
10	9.51	9.36	4.06	2.27		
15	8.04	10.35	7.58	5.43	3.75	
20	15.59	24.18	26.3	17.93	16.34	5.75

[1] Log_e-transformed means of number of ascospores collected in three replicate aliquots, 25 μl, of suspension from nine replicate Petri dish; LSD between log_e-transformed means within time and within temperature were 0.48 and 0.34, respectively.

[2] Back-transformed means of ascospore collected.

3.4 Discussion

Pseudothecia of *L. maculans* developed at 5-20°C and the time taken to reach maturity and discharge ascospores decreased from 5°C to 15°C. The optimum temperature of those tested for pseudothecium development was 15°C. Exposure to a 12 h photoperiod enhanced the production of inoculum by increasing the density of pseudothecia on the stubble compared with darkness. Interrupted wetness prolonged the period of inoculum production, highlighting the importance of wetness for production of pseudothecia and ascospores.

In the present research, the time to reach maturity was significantly longer at lower temperatures than higher temperatures, although only slightly longer at 20°C than 15°C. This supports findings of Pérès *et al.* (1999) that the optimum temperature was 14°C for pseudothecium maturation in a controlled environment and in the field in France. It also agrees with earlier findings in China, that 16°C stimulated formation of pseudothecia of *L. maculans* in a double layer of V8 agar or V8 agar with wheat straw incubated in near UV light (Xu *et al.*, 1987). However, in Britain, Toscano-Underwood *et al.* (2003) reported that the time required for pseudothecia to mature decreased almost linearly with temperature from 5 to 20°C under continuous wetness, both in a controlled environment and in natural conditions. Therefore, the optimum temperature for maturation reported in the British study differs somewhat from that in the present research and in France. In addition, the time to reach stage D at the optimum temperature in this study (22.2 days at 15°C) differed from that in France (14 days at 14°C), but was similar to that in the UK (23.1 days at 20°C). In France, no pseudothecia developed at 10°C (Pérès *et al.*, 1999), whereas in the present study and in the British study (Toscano-Underwood *et al.*, 2003) pseudothecia matured after 40.3 and 40.7 days at this temperature, respectively. In the British study, temperature did not affect the density of pseudothecia, whereas the density was significantly greater at 15°C than 20°C in this research. Differences in methodology, including the means of estimating maturity, age of bble used, environmental conditions in the cabinet, and the pathogen and host genotypes used may have influenced pseudothecium development in the three studies.

The results support previous findings (Pérès *et al.*, 1999) that continuous 95-100% relative humidity is suitable for the development of pseudothecia in a controlled environment. In the

present research, moistening of stubble once a day provided insufficient moisture for the development of pseudothecia at 15°C, whereas moistening twice a day was enough. Maturation took longer under discontinuous wetness than continuous wetness. Three sprays per day significantly decreased the maturation time in comparison with two sprays per day, which agrees with earlier findings (Petrie, 1994) that sporulation occurred earlier in the field as the frequency of moistening of blackleg-affected stubble between April and June increased in Canada. As a result, even at the optimum temperature, 15°C, moisture could influence formation and time to maturity of pseudothecia on stubble, and insufficient moisture inhibited inoculum production. In conclusion, wetness appeared to have a greater influence than the temperatures tested on pseudothecium development. This might explain why epidemics of blackleg often occur in response to winter rainfall, which coincides with susceptibility of young plants to blackleg in Australia (McGee, 1977; McGee & Petrie, 1979).

The frequency of moistening is also important in production and release of inoculum from stubble. As the frequency of moistening increased from two to three times a day, the time taken for pseudothecia to mature (stage D) and discharge ascospores (stage E) at the optimum temperature decreased. This supports the earlier findings of Petrie (1995a) that total rainfall was less important than its frequency in influencing ascospore discharge from blackleg-affected stubble in Saskatchewan. The number of ascospores discharged was negatively related to the number of days from April to June or July having a maximum temperature of 30°C or more (Petrie, 1995a). Irrespective of temperature, ascospore discharge is triggered by moistening of mature pseudothecia on stubble. This supports earlier findings (Petrie, 1995a) that the amount of stubble discharging ascospores, the number of ascospores collected per trapping date and

maximum number of ascospores caught were positively correlated with the number of days with measurable rainfall in the April to July period.

The least mean density of pseudothecia was observed at 20°C, a temperature favourable for growth of most fungi. It is possible that the growth of stubble-associated fungi might contribute to the restricted pseudothecial development on stubble. Although darkness did not affect the taken time for pseudothecia to mature at 15°C, it decreased the density of pseudothecia on stubble. This finding suggests that burying blackleg-affected stubble in soil may reduce pseudothecium formation compared with stubble left on the soil surface and exposed to day/ night photoperiod. This is in contrast to earlier findings that burying of stubble in soil increased pycnidiospore production (Turkington *et al.*, 2000b) and shortened time taken for pseudothecia to develop on buried stubble (Kharbanda & Ostashewski, 1997) compared with surface-placed stubble. The differences between findings may be related to differences in methods, soil characteristics and climatic conditions.

The optimum temperature for ascospore discharge in terms of the amount of ascospores released was 20°C, although peak sporulation occurred earlier at 5-10°C than at 20°C. The longest duration of sporulation was observed at 20°C. No ascospores were captured on slides in trays; however, ascospores were released into Petri dishes with stubble attached to the inside of the lids. The former result is likely to be due to the absence of airflow inside the tray to carry ascospores fast enough to impact on the vertical slides. The latter, simple method may be used to examine ascospore discharge in controlled-environment studies without the need for particular equipment such as a spore liberation tunnel. A similar method was used in the UK to assess ascospore release (Huang *et al.*,

2005), but was published after the method used for the experiment reported here had been developed. Huang *et al.* (2005) reported that temperature did not affect ascospore release from stubble incubated for 4 h at 5-20°C, in contrast to the findings of the present research, and they did not examine the duration of ascospore release. That temperature affected ascospore release in the present research supports earlier findings on ascospore discharge and the inclusion of temperature as a crucial factor in the development of predictive models (Guo & Fernando, 2005; Kruse & Verreet, 2005; McGee, 1977; Pérès *et al.*, 1999).

Pérès *et al.* (1999) reported that the first capture of ascospores was usually observed after rainfall as temperature decreased at the end of summer in France. The optimum temperature for ascospore production on naturally infected stubble was 15°C, although peak sporulation occurred earlier at 20°C and sporulation continued longer at 10°C in Canada (Petrie, 1994), results which differed from those in the present study. These differences may reflect different methods used or variability in the age of the stubble in the different regions. The coincidence of the periods of inoculum production and susceptibility of the crop to infection is favourable for blackleg development in Australia, and the severity of blackleg is related to the amount of inoculum present (McGee & Petrie, 1979; Wherrett *et al.*, 2004). The present study showed that optimum conditions for production of pseudothecia and ascospores were continuous wetness at 15°C in a 12 h photoperiod and the optimum temperature for ascospore discharge over time was 20°C. Such information could be combined with future fieldwork in order to predict the onset of inoculum release. In addition to information about inoculum production, knowledge of appropriate conditions for inoculation and infection of the host plant is required for a better understanding of the disease and the pathogen.

Chapter 4. Germination and hyphal growth from ascospores of *L. maculans* in a controlled environment

4.1 Introduction

In Australia, primary inoculum of *L. maculans* occurs mainly in the form of air-borne ascospores disseminated from pseudothecia on stubble. Germination of ascospores on cotyledons of six canola cultivars began at 2 h after inoculation, and by 4 h 50% and by 6-8 h 85-90% of ascospores had germinated (Hua *et al.*, 2004). Vanniasingham & Gilligan (1988) studied the effects of selected biotic and abiotic factors (temperature, light intensity, wetness period and spore density) on germination of *L. maculans* pycnidiospores. Germination of pycnidiospores was inhibited by high light intensity or by interruption of leaf surface wetness for periods of 20 minutes. However, the experiments were carried out in the absence of host tissues on two nutrient sources, washings of 6-week-old leaves of canola (cv. Primor) and 0.5% pure orange juice (Vanniasingham & Gilligan, 1988). In addition, Vanniasingham & Gilligan (1988) did not investigate the effect of factors mentioned above on the germination of ascospores.

Conn & Tewari (1989) studied the interactions of conidia of *Alternaria brassicae* with leaf epicuticular wax of canola. The rate of germination and number of germ tubes from conidia of *A. brassicae* was greater on canola leaves that had been wiped with a moist cotton swab to remove the wax from the adaxial surface of each leaf, in comparison with those on leaves that had not been wiped. Conidia on cultivars with a greater amount of wax had slower germination and fewer germ tubes than those on cultivars with little wax. It was suggested that the wax layer on the canola leaf may have affected germination by impeding movement of foliar exudates to the leaf surface, as wiping the wax off leaves significantly increased the rate of germination of *A.*

brassicae for all four cultivars tested (Conn & Tewari, 1989). Scanning electron microscopy revealed that germination and hyphal growth from *L. maculans* pycnidiospores were inhibited on leaves of the resistant cultivar Hyola 60 in Australia (Sosnowski *et al.*, 2004). In comparison, germination of and hyphal growth from ascospores of *L. maculans* on leaves of canola cultivars in Australia is little understood.

Ascospores of both L. biglobosa and *L. maculans* germinated at 5 to 20°C on distilled water agar or detached canola leaves (Huang *et al.*, 2001). The percentage of ascospores that germinated and the length of the germ tubes for both species after 24 h of incubation on agar-coated slides increased with rising temperature from 5°C to 20°C. The pattern of hyphal growth, origin and mean number of germ tubes differed between the two groups on agar and on leaves. However, Huang *et al.* (2001) did not investigate the effect of temperature on percentage germination and germ tube elongation on detached cotyledons or leaves of canola. Furthermore, in Western Australia, ascospore germination, penetration and lesion development on cotyledons of six cultivars of spring-type of canola occurred earlier than that with pycnidiospores (Hua *et al.*, 2004). Attachment, germination of ascospores and penetration of cotyledons did not differ between resistant and susceptible cultivars, however, there were marked differences in disease development after penetration. However, Hua *et al.* (2004) did not examine ascospore germination and hyphal growth on the leaf.

Germination of ascospores is an important component of infection of canola by *L. maculans*. Knowledge of the factors that influence the germination of ascospores will result in a better understanding of the factors favouring infection and may lead to the development of more efficient management programs for disease caused by *L. maculans*. The influence of temperature

and cultivar on germination and hyphal growth from ascospores of *L. maculans* on the leaf is poorly understood in Australia. Therefore, the experiments reported in this chapter were designed to study the effects of temperature and cultivar on germination characteristics of ascospores of *L. maculans* applied to cotyledon and leaf of canola in a controlled environment.

4.2 Materials and Methods

4.2.1 Experimental design

The effects of temperature, incubation period, cultivar and plant organ (cotyledon and leaf) on germination characteristics of ascospores of *L. maculans* were examined in the experiment, which was repeated twice. The three repetitions of the experiment were conducted in two incubators (see section 2.2.2) adjusted sequentially to 5°C, 10°C, 15°C or 20°C (Table 4.1). The two incubators were allocated at random for each repetition. Three agar-coated slides, three detached cotyledons and three detached leaves of each cultivar were used per incubation time at each temperature. Prior to inoculation, agar-coated slides, cotyledons and leaves were pre-conditioned for 24 h at the desired temperature. After inoculation, all Petri dishes containing agar-coated slides (one slide per dish), cotyledons (one per dish) and leaves (one per dish) were incubated in darkness at the required temperature and randomly selected for assessment at the end of the designated period of incubation. Percentage germination and germ tube length after 2, 4, 6, 8, 10 and 24 h of incubation at 5-20°C and position and number of germ tubes per ascospore were assessed after 6, 10 and 24 h of incubation at 20°C. The following method was modified from that used by Huang *et al.* (2001).

Table 4.1. Temperatures and parameters assessed at different incubation times on agar-coated slide, canola leaf and cotyledon to investigate the germination of and hyphal growth from ascospores of *Leptosphaeria maculans*.

Germination parameter	Replicate experiment	Temperature (°C)	Period of incubation (h)
Percentage germination	1, 2 & 3	5, 10, 15 & 20	2, 4, 6, 8, 10 & 24
Germ tube length	1, 2 & 3	5, 10, 15 & 20	2, 4, 6, 8, 10 & 24
Position of germ tubes	1, 2 & 3	20	6, 10 & 24
Number of germ tubes	1, 2 & 3	20	6, 10 & 24

4.2.2 Effects of temperature on germination and germ tube elongation of *L. maculans* ascospores on agar-coated slides

In order to examine the effects of temperatures from 5 to 20°C on germination characteristics of *L. maculans*, ascospore suspensions and agar-coated slides were prepared and assessed as described below.

4.2.2.1 Preparation of ascospore suspension

Pieces of tap root and stem base (*ca* 20-30 cm in length) of one-year-old canola stubble with blackleg cankers were obtained from a blackleg disease nursery in June 2003 (see section 2.1.3). The identity of ascospores obtained from this material was confirmed by microscopic examination. To produce mature pseudothecia and ascospores, cankered stem pieces were soaked in distilled water over night and then placed in plastic trays (45 × 31 × 5 cm) lined with Chux cloth (60 × 30 cm, Clorox Pty Ltd, Australia), as described in section 3.2.1. Trays were then

incubated at 15°C under a 12 h photoperiod (see section 2.2.2) for 3 weeks to minimize variation between pseudothecium maturation stages and ascospore age. To confirm the maturity of pseudothecia and ascospores, approximately three mature psedothecia were excised from 10 stems at random and examined by microscope.

Fresh ascospore suspensions were used as inoculum in controlled environment experiments. To prepare suspensions, 3-6 cm lengths of stubble bearing mature *L. maculans* pseudothecia and ascospores were attached to the underside of 9 cm diameter Petri dish lids with Vaseline. The pieces of stubble were immersed in distilled water for 15 s to swell the asci, then the water was poured off and the lid placed over the Petri dish base. After 1 h at room temperature (approximately at 22°C) followed by 1 h at 4-5°C, large numbers of ascospores were released into the dishes. Sterile distilled water (5 ml) was added to each Petri dish and the ascospores were suspended using a sterile artist's paint brush. Ascospore suspensions from a number of dishes were combined and the concentration was adjusted to 5×10^3 ascospores ml^{-1} with the aid of a haemocytometer.

4.2.2.2 Preparation and inoculation of agar-coated slides

Molten 1.5% distilled water agar (WA, appendix 2), 0.5 ml, was placed on the centre of each sterile glass slide (76.2 × 25.4 × 0.8-1.0 mm, Livingstone, Australia) using an Eppendorf pipette. The agar was spread uniformly to form a continuous layer of *ca* 4 × 1.5 cm. The agar was inoculated by applying 40 μl of fresh ascospore suspension onto each slide using an Eppendorf pipette. Each slide was then placed in a Petri dish (9 cm diameter) containing two layers of filter paper (Whatman no. 1; Whatman International Ltd, Maidstone, UK) moistened with sterile

distilled water. The Petri dishes were then placed in incubators at the designated temperature and germination characteristics were assessed after designated times.

4.2.2.3 Assessment of ascospore germination and germ tube elongation

After the designated time, agar slides were removed from the incubators and two drops of trypan blue (0.1% w/v in lactophenol) were immediately spread onto the surface to stop growth. The percentage of ascospores that had germinated was determined by assessing the first 100 ascospores observed on each slide, using a light microscope (Olympus BH-2, Japan) at 250 × magnification. Ascospores were considered to have germinated if the length of the germ tube exceeded the width of ascospore (*ca* 5 μm). The length of the germ tube from 20 ascospores per slide was measured at all temperatures and incubation times tested. If the ascospore had produced more than one germ tube, only the longest one was measured.

4.2.3 Effects of temperature and cultivar on germination and germ tube elongation of *L. maculans* ascospores on plant tissue

Germination characteristics for *L. maculans* ascospores were observed on detached leaves and cotyledons of five canola cultivars, Hyola 60, Ripper, Monty, Karoo and Q2, differing in resistance to blackleg disease (see Table 2.1). Three replicate experiments at 5°C, 10°C, 15°C or 20°C were conducted sequentially in two temperature-regulated incubators (Table 4.1). Three leaves or cotyledons, with one inoculation site on each, were used per cultivar, duration of incubation and temperature treatment.

4.2.3.1 Inoculation of leaf and cotyledon

Plants were grown as described in section 2.1.2. The day before inoculation, cotyledons were detached from plants at GS 0, and first and second leaves were detached from plants at GS 1.3 (appendix 1). Cotyledons and leaves were placed, adaxial side up, in 9 cm Petri dishes (one leaf or one cotyledon per dish) lined with two layers of filter paper (Whatman no. 41; Whatman International Ltd, Maidstone, UK) moistened with sterile distilled water and incubated 24 h at the desired temperatures. Leaves and cotyledons were then inoculated with ascospore suspension prepared as described in section 4.2.2.1. One site on the adaxial surface of each leaf or cotyledon was inoculated by applying one drop (20 µl) of fresh ascospore suspension using an Eppendorf pipette. Petri dishes were incubated in darkness at the required temperature until the end of each designated incubation time.

4.2.3.2 Assessment of ascospore germination and elongation of germ tubes

After the designated incubation time, leaves and cotyledons were removed from the two incubators and one drop of trypan blue (0.1% w/v in lactophenol) was placed on each inoculation site to stop growth, and then placed in a fume hood to dry. A drop of collodion (30% in absolute ethyl alcohol, BDH Laboratory Supplies, Poole, England) was placed on each inoculation site and left to dry for 5 minutes. Each collodion membrane was then peeled off with forceps and placed in a drop of distilled water on a glass slide. Germination characteristics of ascospores on collodion membranes from detached leaves and cotyledons of all five canola cultivars were assessed as above (see section 4.2.2.3).

4.2.4 Effect of duration of incubation and cultivar on hyphal growth

As the hyphal growth of ascospores was greatest at 20°C, the total number of germ tubes per ascospore and the origin of the germ tubes, whether from terminal or from interstitial cells, were assessed at 20°C after 6, 10 and 24 h of incubation. The patterns of hyphal growth from ascospores on representatives were photographed after 2, 8 and 24 h of incubation at 20°C.

4.2.5 Statistical analysis

The data for the number of germinated ascospores on agar-coated slides, leaves and cotyledons of the five cultivars at the four temperatures tested in the three repetitions of the experiment were converted to percentage and subjected to ANOVA (see section 2.4) using a split-plot analysis with temperature as main block. The time to reach 50% germination of ascospores (T_{50}) and rate of germination per hour (R_{50}) at T_{50} were obtained from the data for each temperature. The equation used to calculate the rate of germination was as follows:

R_{50} = Number of ascospores when 50% germinated / T_{50} at each temperature (h)

The data for the length of germ tubes on agar-coated slides, leaves and cotyledons of the five cultivars at the four temperatures tested in the three repetitions of the experiment were analysed together using a split-plot analysis with temperature as main block. ANOVA was used to assess the effects of time, plant organ and cultivar on the total number of germ tubes per ascospore and the ratio of the number of terminal to interstitial germ tubes from ascospores on agar slide, leaf and cotyledon of the five canola cultivars at 20°C. The data for the ratio of the number of terminal to interstitial germ tubes were $\log_e$-transformed before analysis to improve the

homogeneity of data. Mean values for $\log_e$-transformed data were calculated and back-transformed. The data were analysed by K. Dowling, BiometricsSA, South Australia. The data for the length of germ tubes on agar-coated slides from the three repetitions of the experiment on agar-coated slides over time at 5-20°C were used for linear regression line.

4.3 Results

4.3.1 Effect of temperature and cultivar on ascospore germination

The four-way interaction of temperature, incubation time, cultivar and plant organ (cotyledon and leaf) was not significant ($P = 0.5$, see appendix 3), whereas two-way interactions of temperature and incubation time and of cultivar and plant organ significantly affected ($P < 0.001$) the mean percentage germination of *L. maculans* ascospores on plant tissues. Therefore, data for these significant interactions only are presented here.

Germination was first observed after 2 h at 10-20°C and 4 h at 5°C (Table 4.2). After 2 h of incubation, the mean percentage germination significantly increased (LSD = 5.1; $P < 0.05$) from 9.5% at 10°C to 24.6% at 15°C and to 35.7% at 20°C. Germination increased ($P < 0.05$) with increasing incubation time from 2 to 24 h at 5 and 10°C, whereas this factor increased little ($P > 0.05$) over 10-24 h at 15°C and over 8-24 h at 20°C. After 24 h of incubation, the mean percentage germination was similar ($P > 0.05$) between 5 and 10°C and the same between 15 and 20°C, regardless of cultivar and plant organ. Germination was greater ($P < 0.05$) at 15 and 20°C than at 5 and 10°C over time.

Table 4.2. Effects of temperature and incubation time on mean percent germination of *Leptosphaeria maculans* ascospores from three repetitions of the experiment on agar-coated slide, detached cotyledon and leaf of five canola cultivars.

Time (h)	Temperature (°C)			
	5	10	15	20
2	0[1]	9.5	24.6	35.7
4	1.6	31.7	62.1	63.8
6	10.5	47.9	74.1	82.2
8	21.4	60.4	81.2	87.3
10	39.1	66.8	89	88.5
24	81.1	82.6	90.5	90.5

[1] Means of germination of ascospores on agar-coated slides, cotyledons and leaves of the five cultivars combined for all time intervals; LSD = 5.1; $P < 0.05$.

Table 4.3 shows the overall means for germination of ascospores on agar-coated slides, cotyledons and leaves for all cultivars at all time intervals. The mean percentage germination was significantly greater (LSD = 1.6; $P < 0.05$) on agar-coated slides than on leaves of all five cultivars (Table 4.3). Germination was greater ($P < 0.05$) on agar-coated slides and on cotyledons of Karoo and Q2 than on cotyledons of Hyola 60 and Ripper. On leaves, germination was greater ($P < 0.05$) on Karoo and Q2 than Hyola 60 and Ripper. For each cultivar, significantly more germination ($P < 0.05$) occurred on cotyledons than on leaves, regardless of temperature and incubation time.

Table 4.3. Effects of cultivar and substrate on mean percent germination of *Leptosphaeria maculans* ascospores from three repetitions of the experiment after 2-24 h incubation time at 5-20°C.

Cultivar	Substrate		
	Water agar	Detached cotyledon	Detached Leaf
Hyola 60		55.2[1]	50.5
Ripper		54.7	49.7
Monty		56.8	53.7
Karoo		57.5	55
Q2		59.2	55.7
Agar-coated slide	57.9		

[1] Means of germination of ascospores combined for incubation time 2-24 h at 5-20°C; LSD = 1.6; $P < 0.05$.

Table 4.4. Mean percent germination of *Leptosphaeria maculans* ascospores from three repetitions of the experiment on detached cotyledon and leaf of five canola cultivars after 2-24 h of incubation time at 5, 10, 15 & 20°C.

Time (h)	Cultivar	Temperature (°C)							
		5		10		15		20	
		C	L	C	L	C	L	C	L
2	Hyola 60	0[1]	0	9	7	25	21	37	31
	Ripper	0	0	12	6	21	19	39	30
	Monty	0	0	12	8	29	25	39	33
	Karoo	0	0	12	10	26	25	38	31
	Q2	0	0	11	7	27	24	39	34
4	Hyola 60	2	0	31	26	62	52	65	62
	Ripper	1	0	31	18	53	49	62	62
	Monty	1	1	31	32	65	62	66	60

	Karoo	2	1	34	34	70	65	65	63
	Q2	3	2	38	33	70	66	66	61
6	Hyola 60	12	6	50	39	66	69	83	80
	Ripper	10	4	47	41	71	71	82	79
	Monty	12	9	48	47	78	74	81	79
	Karoo	13	10	49	51	79	76	85	80
	Q2	15	11	52	51	81	74	88	82
8	Hyola 60	20	17	59	50	82	80	88	80
	Ripper	23	13	61	53	81	81	88	80
	Monty	23	20	65	55	81	81	91	86
	Karoo	23	22	59	60	83	80	90	88
	Q2	30	22	68	67	82	81	91	89
10	Hyola 60	45	29	65	59	90	87	90	80
	Ripper	43	23	60	59	90	87	91	85
	Monty	44	35	69	65	90	89	89	86
	Karoo	48	33	71	71	90	88	90	88
	Q2	49	35	72	71	92	88	92	91
24	Hyola 60	81	80	81	79	90	90	90	88
	Ripper	81	76	84	78	90	90	91	87
	Monty	83	82	82	80	91	90	91	91
	Karoo	83	82	85	84	91	90	92	88
	Q2	83	81	87	84	92	90	93	92

C = detached cotyledon; L = detached leaf.

[1] LSD = 9; P > 0.05.

Although the four-way interaction of temperature, incubation time, cultivar and plant organ was not statistically significant, means of percentage germination of ascospores on cotyledons and leaves of the five cultivars over time are presented in Table 4.4 to allow the trends to be examined. After incubation for 24 h at each temperature, the mean percentage germination was

similar ($P > 0.05$) between cotyledons and leaves, and between the five cultivars, except for greater germination ($P < 0.05$) on cotyledons of Q2 than on leaves of Ripper at 10°C.

The mean observed maximum percentage germination, which occurred after 24 h at each temperature, tended to increase from 81.1% at 5°C to 90.5% at 20°C (Table 4.5). The time for 50% of ascospores to germinate (T_{50}) was 13.1 h at 5°C and 2.5 h at 20°C. The rate of germination per hour (R_{50}) at T_{50} increased from 6.2 at 5°C to 36.2 at 20°C.

Table 4.5. Maximum percentage germination, time to 50% germination and rate of germination of ascospores of *Leptosphaeria maculans* on agar slides, detached cotyledon and leaf of five canola cultivars[1] at 5, 10, 15 & 20°C.

Germination parameter	Temperature (°C)			
	5	10	15	20
G_{max}[2]	81.1	82.6	90.5	90.5
T_{50}[3]	13.1	6	3	2.5
R_{50}[4]	6.2	13.8	30.2	36.2

[1] Data for substrates and cultivars were combined.

[2] The observed maximum mean percentage of ascospores that had germinated.

[3] The observed time (h) for 50% of ascospores to germinate.

[4] The observed rate of germination per hour.

4.3.2 Effect of temperature and cultivar on germ tube elongation

4.3.2.1 On agar-coated slides

The interaction of temperature and incubation time affected ($P < 0.001$) the mean length of germ tubes from ascospores of *L. maculans* on agar-coated slides (Figure 4.1). After incubation for 2 h,

germ tubes on agar-coated slides at 5°C were significantly shorter (LSD = 6.7; $P < 0.05$) than those at 10-20°C. After incubation for 4-6 h, the mean length of germ tubes was greater ($P < 0.05$) at higher temperatures than lower ones, except between 10 and 15°C. Germ tubes were longer ($P < 0.05$) after incubation for 8-24 h at higher temperatures than lower ones.

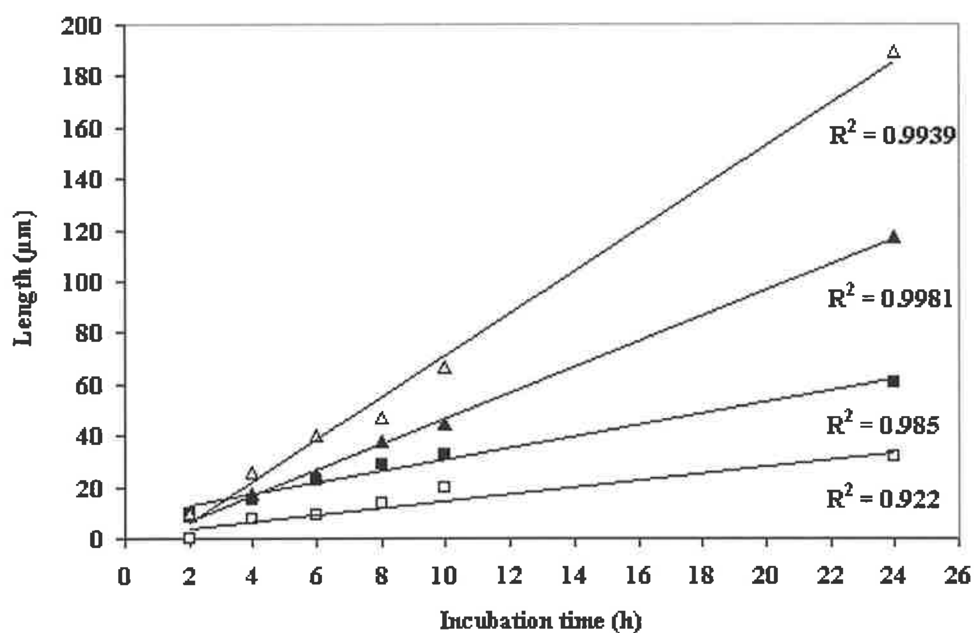


Figure 4.1. The mean length (µm) of germ tubes from ascospores of *Leptosphaeria maculans* from the three repetitions of the experiment on agar-coated slides over time at 5 (□), 10(■), 15(▲) and 20°C (△); LSD = 6.7; $P < 0.05$; linear regression line and R-squared value are presented for each temperature.

The equations of the regression lines of the length of germ tubes of ascospores on incubation time in Figure 4.1 at different temperatures (temperature shown as subscript) are as follows:

$Y_5 = 1.3438x + 1.4277$

$Y_{10} = 2.2706x + 7.9642$

$Y_{15} = 4.9748x - 2.8335$

$Y_{20} = 8.176x - 10.389$

The equations did not change when the regression lines went through the origin

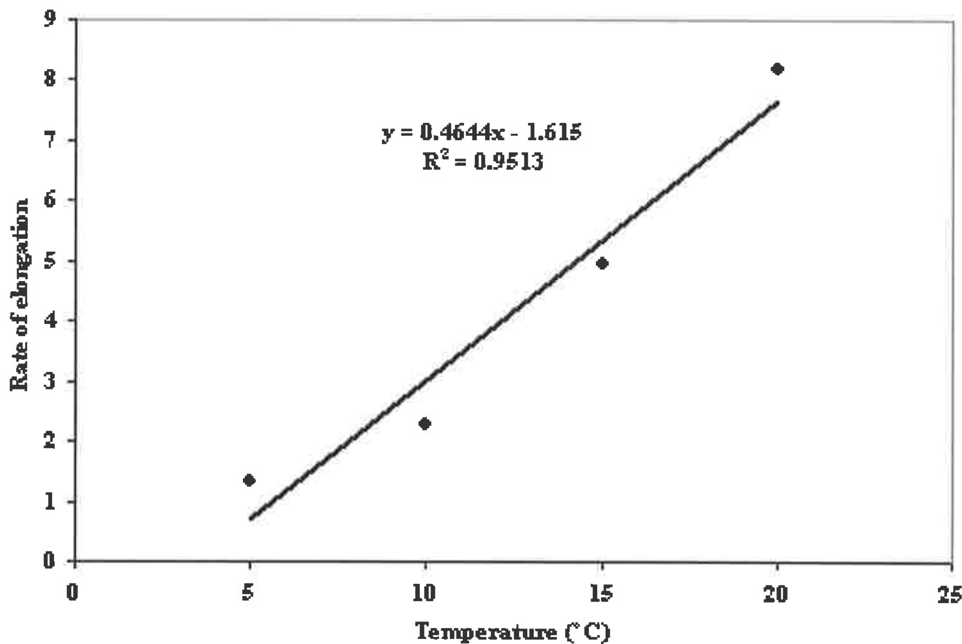


Figure 4.2. The rate of elongation of germ tubes from ascospores of *Leptosphaeria maculans* on agar-coated slides at 5, 10, 15 and 20°C presented as the regression line, the equation and R-squared value; data points are coefficient of x in the following equations for each temperature (as subscript): $Y_5 = 1.3438x + 1.4277$, $Y_{10} = 2.2706x + 7.9642$, $Y_{15} = 4.9748x - 2.8335$, and $Y_{20} = 8.176x - 10.389$.

The elongation rate of germ tubes from ascospores germinated on agar-coated slides at each temperature was obtained from the above-mentioned equations, using the coefficient of x for

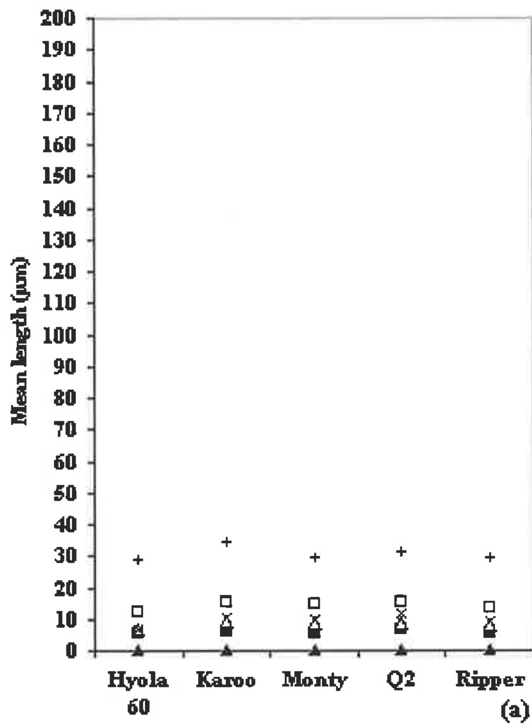
, which was zero at 3.5°C,

each temperature (Figure 4.2). The rate of elongation increased with increasing temperature from 5 to 20°C and nearly doubled for every 5°C increase in temperature.

4.3.2.2 On plant tissue

The four-way interaction of temperature, incubation time, cultivar and plant organ (cotyledon or leaf) significantly affected ($P = 0.03$) the length of germ tubes from ascospores of *L. maculans* on detached leaves and cotyledons of canola. After incubation for 24 h, the mean length of germ tubes on cotyledons and leaves of the five cultivars significantly increased (LSD = 6.7; $P < 0.05$) with increasing temperature from 5 to 20°C, except on cotyledons of Hyola 60, on which longer ($P < 0.05$) germ tubes were observed at 15°C than those at 20°C (Figure 4.3).

The mean length of germ tubes from ascospores was generally greater on cotyledons and leaves of susceptible cultivars than resistant cultivars after incubation for 24 h at 20°C. At 5°C, there was no significant difference ($P > 0.05$) in the mean length on cotyledons and leaves between the cultivars over time (Figure 4.3a, b). At 10°C, germ tubes were significantly longer ($P < 0.05$) on leaves of Karoo than of Ripper after 6 h and longer on cotyledons and leaves of Q2 or on cotyledons of Karoo than Ripper after 24 h (Figure 4.3c, d). Longer germ tubes were observed on cotyledons of Hyola 60 than of the other cultivars after 24 h at 10°C. At 15°C, germ tubes were significantly shorter ($P < 0.05$) on leaves of Ripper than of Karoo after 2 h, shorter on leaves of Ripper and Hyola 60 than of Karoo, Q2 and Monty after 8 h, and also shorter on cotyledons or leaves of Ripper than of the other cultivars after 10-24 h (Figure 4.3e, f). At 20°C, germ tubes were generally longer on cotyledons and leaves of Q2 and Karoo than of Hyola 60 and Ripper after from 4 to 24 h (Figure 4.3g, h).


Mean length (µm)
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170
160
150
140
130
120
110
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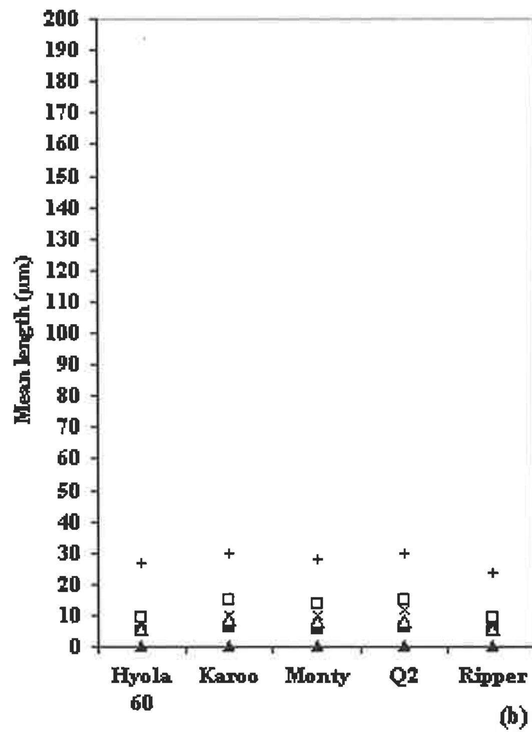
30
20
10
0
Hyola 60
Karoo
Monty
Q2
Ripper
(a)


Mean length (µm)
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Ripper
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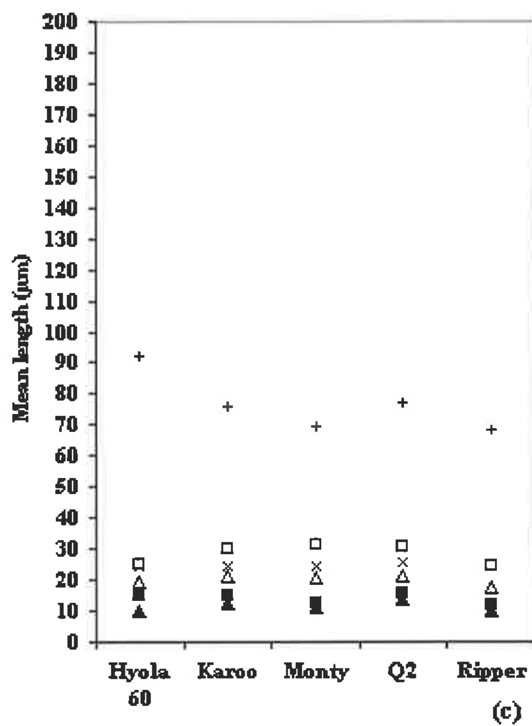
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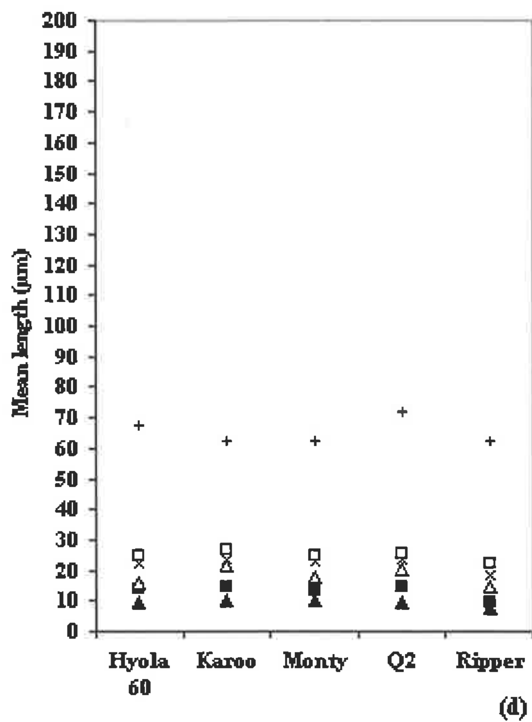
Mean length (µm)
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Hyola 60
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Ripper
(d)

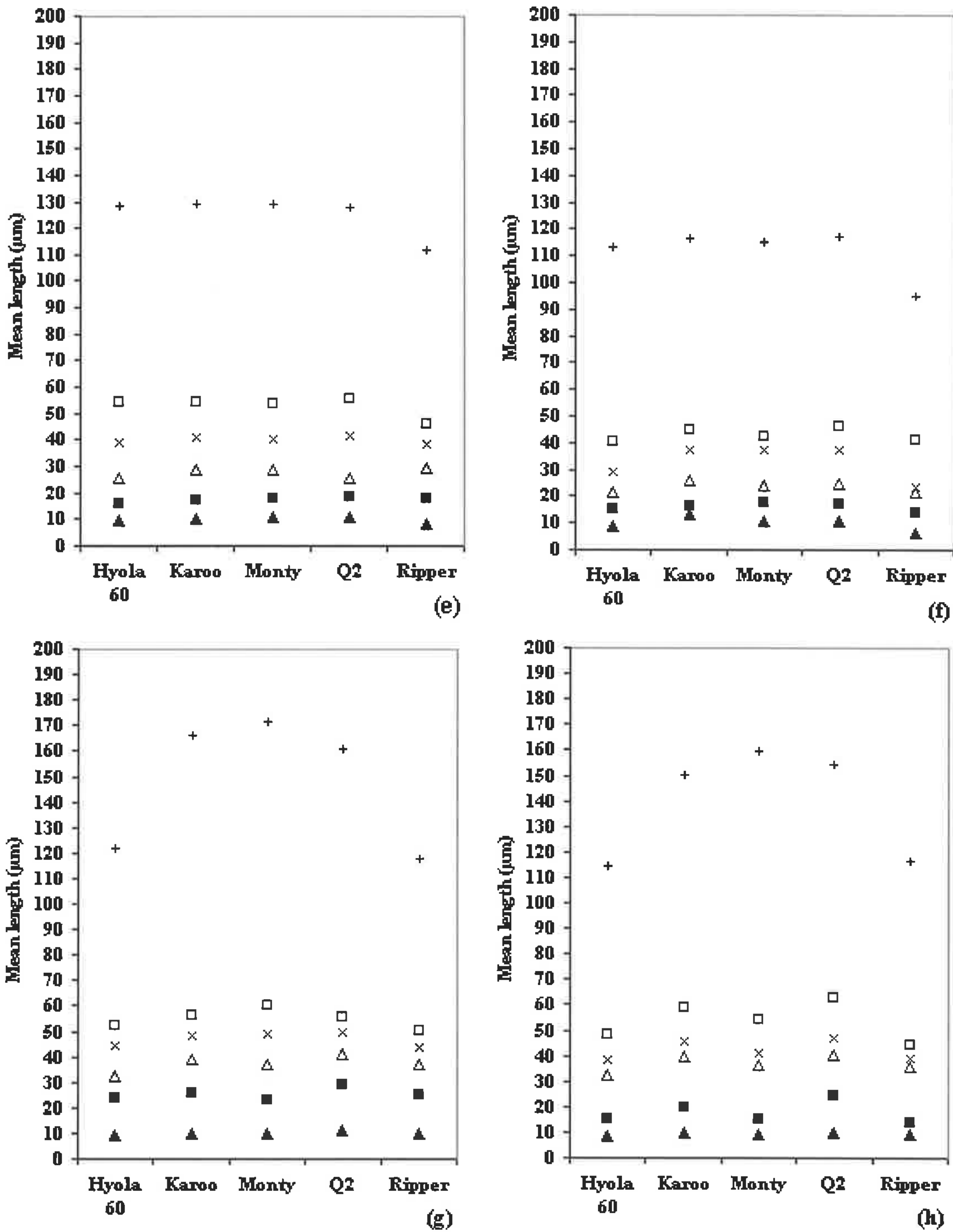


Figure 4.3. The mean length (µm) of germ tubes from ascospores of *Leptosphaeria maculans* from the three repetitions of the experiment on detached cotyledons (a, c, e, g) and leaves (b, d, f, h) of five canola cultivars after 2 (▲), 4 (■), 6 (△), 8 (×), 10 (□) and 24 (+) h incubation at 5 (a, b), 10(c, d), 15(e, f) & 20°C (g, h); LSD = 6.7; P < 0.05.

In general, elongation of germ tubes from ascospores was greater on cotyledons than on leaves of the cultivars after incubation for 24 h at 10-20°C. At 10°C after 24 h, germ tubes were significantly longer ($P < 0.05$) on cotyledons of Hyola 60 (92 μm) and Karoo (75.8 μm) than those on the corresponding leaves (67.4 and 62.2 μm). At 15°C after 6 h, longer ($P < 0.05$) germ tubes on cotyledons (29.2 μm) than leaves (21.8 μm) occurred only on Ripper, however, after 24 h, elongation was greater ($P < 0.05$) on cotyledons than leaves of all cultivars. At 20°C, germ tubes on cotyledons were significantly longer ($P < 0.05$) than leaves of most cultivars only after 4 and 24 h.

Table 4.6. The mean number of germ tubes per ascospore and the ratio of terminal to interstitial germ tubes (T/I) from the three repetitions of the experiment on agar-coated slides, detached cotyledons and leaves of five canola cultivars[1] after 6, 10 and 24 h incubation at 20°C.

Germination parameter	Incubation time (h)			LSD
	6	10	24	
Number of germ tubes per ascospore	2.4	3.1	3.6	0.4
T/I[2]	0.3	-0.1	-0.2	0.2
T/I[3]	1.4	0.9	0.8	

[1] Data for substrate and cultivar were combined as there were no significant difference.

[2] Loge-transformed means (Log_e (T/I)); $P < 0.05$.

[3] Back-transformed means.

Maximum mean length of germ tubes was always observed on cotyledons of all cultivars after 24 h at all temperatures tested. The longest germ tubes were observed on cotyledons of Monty (171.7 μm) after incubation at 20°C for 24 h.

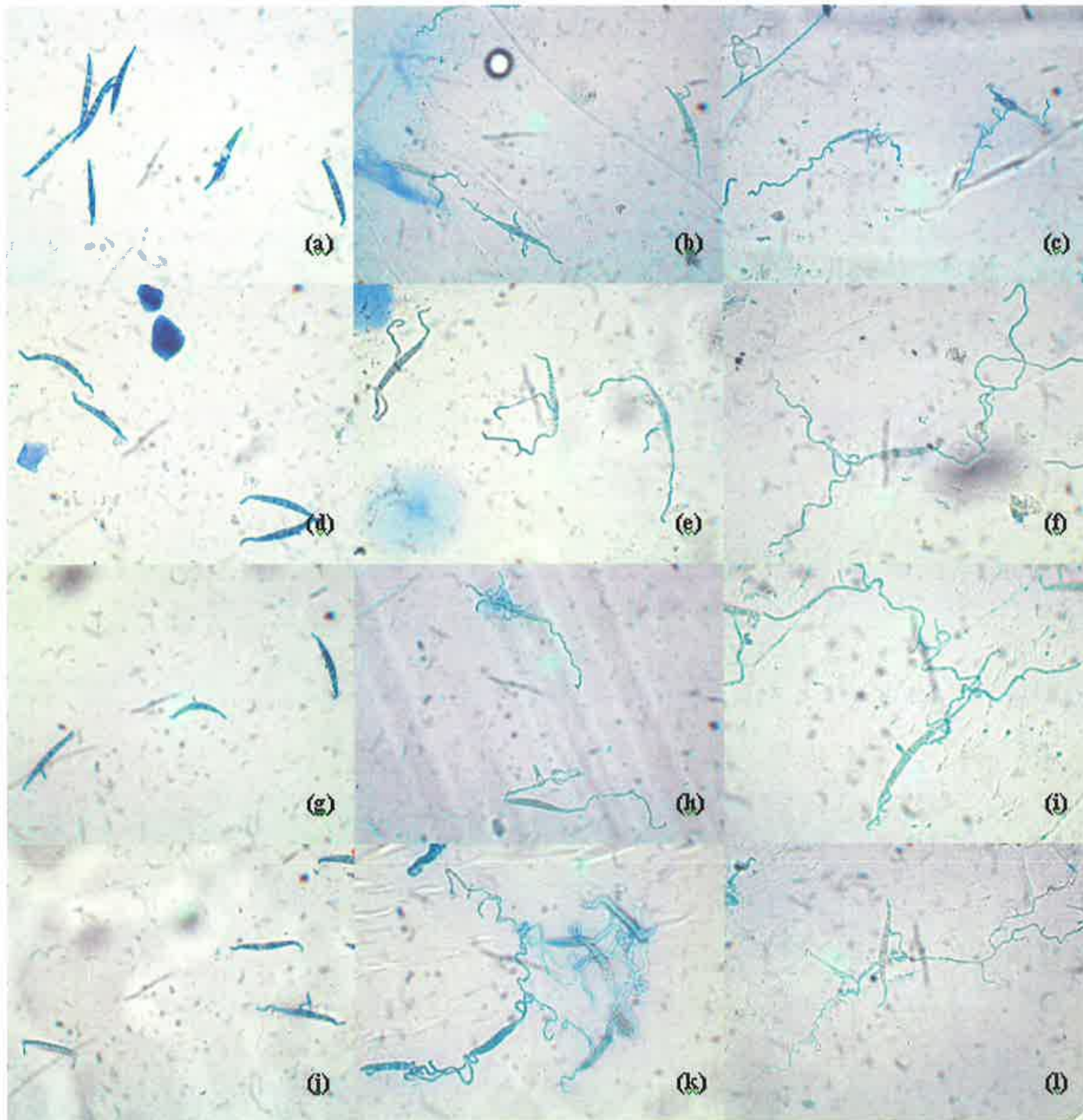


Figure 4.4. Patterns of hyphal growth from ascospores of *Leptosphaeria maculans* germinated on detached leaves (a-f) and cotyledons (g-l) of one resistant cultivar (a-c & g-i) and one susceptible cultivar (d-f & j-l) as representatives of all five canola cultivars at 20°C after 2 (a, d, g, i), 8 (b, e, h, k) and 24 (c, f, i, l) h of incubation.

4.3.3 Effect of duration of incubation and cultivar on hyphal growth

Cultivar did not significantly affect the mean number of germ tubes (P = 0.4) and the ratio of terminal to interstitial germ tubes (P = 0.7) from ascospores at 20°C (data not shown). Additionally, no significant difference was observed between mean number of germ tubes and the ratio of terminal to interstitial germ tubes from ascospores on agar-coated slides, cotyledons and leaves at the same temperature (data not shown). The mean number of germ tubes per ascospore significantly increased (LSD = 0.4; $P < 0.05$) from 2.4 to 3.6 with increasing incubation time from 6 to 24 h at 20°C (Table 4.6). The ratio of terminal to interstitial germ tubes significantly decreased (LSD = 0.2; $P < 0.05$) with increasing incubation time from 6 to 10 h and then changed little over the next 14 h.

After 10 h incubation at 15-20°C, germ tubes had grown and branched extensively, forming a combination of straight and convoluted hyphae that were difficult to measure. The pattern of hyphal growth did not differ on agar-coated slides, detached cotyledons and leaves. There was no obvious difference in patterns of hyphal growth from ascospores germinating on the various cultivars tested (Figure 4.4a-l).

4.4 Discussion

These experiments provide detailed information on germination characteristics of ascospores of *L. maculans* on agar-coated slides, cotyledons and leaves of the canola cultivars tested. Results support earlier findings (Huang *et al.*, 2001) that germination begins after 2 h at 10-20°C and 4 h at 5°C on distilled water agar. Whereas Huang *et al.* (2001) reported that germination percentage after 24 h increased with increasing temperature from 5-20°C on agar-coated slides, in the present

study, germination of ascospores after 24 h on agar-coated slides, detached cotyledons and leaves of the five canola cultivars tested was similar between 5 and 10°C and the same between 15 and 20°C. The maximum percentage germination of ascospores obtained in the present study, 81.1, 82.6, 90.5 and 90.5%, was greater than 65, 75, 80 and 89% presented by Huang *et al.* (2001) at 5, 10, 15 and 20°C, respectively. Furthermore, the time taken for 50% of ascospores to germinate at 10-20°C in the present study was shorter than that in the British study (13.1, 6, 3 and 2.5 in the present study *cf* 8.9, 7.7, 5.7 and 6.8 h in the British study), whereas germination took longer at
differences in environmental conditions (temperature, light, humidity) within growth cabinets or, perhaps,
5°C. The difference between the two studies suggests variability in the population of the pathogen from different geographical locations.

The optimum temperature for germination of ascospores of *L. maculans* on detached cotyledons and leaves of the canola cultivars tested was 15-20°C, at which maximum germination occurred. Therefore, the optimum temperature range for ascospore germination is similar for ascospores on agar and on plant tissue. Ascospore germination was greater on agar-coated slides and cotyledons than on leaves of all cultivars tested, regardless of temperature and incubation time, suggesting that agar and cotyledons provide an environment more conducive to germination than do leaves. If this finding is confirmed, then further investigation is warranted to determine the basis for such differences.

This research revealed that mean percentages of ascospore germination were greater on leaf or cotyledon of the susceptible cultivars, Q2 and Karoo, than those of resistant cultivars, Hyola 60 and Ripper, regardless of temperature and incubation time. Likewise, greater germination occurred on leaf or cotyledon of Q2 than those of Monty. Although germination of ascospores on the cotyledon did not differ between resistant and susceptible cultivars in Western Australia (Hua

et al., 2004), germination and hyphal growth from pycnidiospores of *L. maculans* were inhibited on the resistant cultivar Hyola 60 in South Australia (Sosnowski *et al.*, 2004). The differences in findings in South Australia and those in Western Australia might be due to different pathogen or host plant genotypes or methodology and further research may elucidate the basis of such differences. However, such small differences observed in the present research, even statistically significant, are unlikely to influence the development of blackleg in the field.

As suggested earlier (Sosnowski *et al.*, 2001b), further research in breeding for resistance to blackleg at the cotyledon and leaf level is required. Plant surface structures and chemicals can strongly influence the susceptibility of a plant to disease (Nicholson & Epstein, 1991). Therefore, further research is required to determine if slightly reduced germination of ascospores on resistant cultivars might contribute to disease resistance and to identify leaf surface structures and biochemicals that may contribute to these differences.

Huang *et al.* (2001) showed that the length of germ tubes for ascospores of L. maculans and L. biglobosa on agar-coated slides increased with increasing temperature from 5°C to 20°C. The present study has provided additional data on the elongation of germ tubes from ascospores of *L. maculans* on detached cotyledons and leaves of canola cultivars differing in blackleg-resistance rating and enabled comparisons to be made among germination parameters for cotyledons and leaves and among cultivars. Generally longer germ tubes on the susceptible cultivars, Q2 and Karoo, than the resistant cultivars, Hyola 60 and Ripper, after 24 h at 20°C supports earlier findings using scanning electron microscopy (Potter *et al.*, 2003) of restricted germination and hyphal growth from pycnidiospores of *L. maculans* on leaves of the resistant cultivar, Hyola 60. However, the effect was not consistent at all temperatures and incubation times examined here.

Although elongation of germ tubes was generally greater on cotyledons than on leaves of the cultivars tested and on susceptible cultivars than resistant cultivars after 24 h at 15-20°C, there was little difference in the mean length of germ tubes between leaves and cotyledons of the five cultivars at 5°C. This suggests that temperature has a greater influence on germination of and hyphal growth from *L. maculans* ascospores than duration of incubation, cultivar or plant organ (cotyledon or leaf), as cultivar and plant organ had no significant effect on the elongation of germ tubes at 5°C. Chilling at 5°C probably overrides all other factors tested, whereas, increasing the temperature to 15-20°C allowed cultivar or plant organ (cotyledon or leaf) to have an effect. Increasing temperature may activate chemicals or affect surface structures on cotyledon or leaf, such as epicuticular wax, which may affect fungal growth. In addition, examination of germination of ascospores and germ tube elongation on detached cotyledon or leaf might be used to assess the resistance of new cultivars in the laboratory, particularly to screen for leaf resistance.

In summary, analysis of data from the above experiments suggested that temperature had a greater influence than plant organ (cotyledon and leaf) and cultivar, on germination and elongation of germ tubes from ascospores. This information contributes to the understanding of the pre-penetration stage of infection of canola by *L. maculans*. In addition, further information on the processes of infection is required to inform management strategies for blackleg disease.

Chapter 5. Incubation period of *L. maculans* on canola in a controlled environment

5.1 Introduction

L. maculans invades young leaves and cotyledons via stomata or wounds and colonizes intercellular spaces between mesophyll cells, which finally results in the death of tissues. Phoma leaf spots are dead tissues on which pycnidia appear (Hammond & Lewis, 1987; Hammond *et al.*, 1985). After colonization of leaves, the pathogen grows down the petiole to reach the stem. Severe basal stem cankers originate from infection of leaves that occurs at early stages of plant growth (see section 1.3.1). The incubation period, which is described as the time from inoculation of leaves to the appearance of the first lesions (Biddulph *et al.*, 1999b), is one of the early stages of the disease cycle. Accurate data on incubation period and, indeed, all stages of disease development are required for a better understanding of blackleg disease.

Infection and the duration of the incubation period are influenced by environmental conditions. Hall (1992) suggested that wetness is more important than temperature for infection by *L. maculans*. In the UK, Biddulph *et al.* (1999b) showed that the duration of wetness period affected the length of the incubation period only at sub-optimal temperatures and that most lesions appeared following 48 h of wetness at 5-20°C. Toscano-Underwood *et al.* (2001) found that the length of the incubation period of *L. maculans* on cv. Lipton, moderately resistant to blackleg, following 48 h of wetness increased from 3 to 15.5 days, as temperature decreased from 20 to 5°C. In Australia, Sosnowski *et al.* (2003) showed that leaves inoculated with pycnidiospores produced lesions 25 days after inoculation at 8°C. The most lesions appeared on inoculated leaves exposed to a day/night temperature of 18/15°C following 96 h of wetness (Sosnowski *et*

al., 2005). The variation among these studies suggests that factors such as type of inoculum used (ascospores or pycnidiospores), variability in *L. maculans* populations from different geographical locations, and cultivar may influence the length of the incubation period on canola. Also, there is a need to investigate the incubation period on canola plants inoculated with ascospores, which are the major primary inoculum in Australia (McGee & Petrie, 1979). Observations on blackleg development in Australia suggest that the differences in severity of epidemics between seasons and between sites may be related to the occurrence of weather conditions favourable for infection of plants by ascospores, particularly differences in rainfall and temperature. To optimize sowing date, choice of cultivar and fungicide use, there is a need to determine the conditions when ascospores of *L. maculans* can infect and produce lesions on leaves and cotyledons of canola in south-eastern Australia.

Leaf position or plant age appeared to influence infection in some host-pathogen interactions. The effect of leaf position on race-nonspecific resistance against *Phytophthora infestans* was more important than plant or leaf age, and apical leaves were more resistant to late blight than basal leaves in both field and controlled environment (Visker *et al.*, 2003). Likewise, the development of leaf blight (caused by *Bipolaris coicis*) on six adlay (*Coix lacryma-jobi* L.) cultivars of differing resistance increased with the ageing of plants and leaves (Chang & Hwang, 2003). However, incubation period of *L. maculans* was shortened by increasing temperature, relative humidity and photoperiod, and by wounding, but was not affected by plant age, cultivar or pycnidiospore density (Vanniasingham & Gilligan, 1989). In contrast, previous research indicated that canola was most susceptible to blackleg prior to the 6-leaf stage of growth (Alabouvette & Brunin, 1974; McGee & Petrie, 1979), and infection following inoculation with pycnidiospores and subsequent development of leaf and stem lesions were influenced by cultivar

resistance (Hua *et al.*, 2006; Sosnowski *et al.*, 2004). Both severity and incidence of blackleg were affected by the growth stage (Hua *et al.*, 2006) and only plants inoculated, with pycnidiospores, at the cotyledon and 3-leaf stages developed severe blackleg or died (Marcroft *et al.*, 2005). There was no difference between near-isogenic isolates of *L. maculans* differing at the *AvrLm4* avirulence locus (*AvrLm4* or *avrLm4*) in incubation period on the leaf of cvs. Eurol and Darmor (both lacking the resistance gene *Rlm4*) inoculated with ascospores and pycnidiospores (Huang *et al.*, 2006b). However, no comparison in terms of incubation period was made between resistant and susceptible cultivars. Furthermore, the susceptibility of three canola cultivars to Phoma leaf spot was examined at three vegetative stages (with 2, 4 and 6 leaves) inoculated with ascospores from stubble placed adjacent to the seedlings in a plastic tunnel in the field at 8-14°C (Poisson & Pérès, 1999a). The time to appearance of leaf spots was longer for plants inoculated at the 2-leaf stage than 6-leaf stage for all cultivars and the reaction at the 4-leaf stage was dependent on cultivar. Leaf lesions appeared later on the tolerant cultivar than susceptible cultivars. Poisson & Pérès (1999a) suggested that incubation period might differ among cultivars and among leaves at different positions. However, they looked at the time from placing of moistened stubble in the tunnel to the appearance of first leaf spots, which cannot be considered as incubation period, as it also included time required for the release of ascospores from mature pseudothecia and inoculation. It would appear that the effect of leaf position and cultivar on incubation period of *L. maculans* on canola plants inoculated with ascospores is not well defined.

There are many reports of the variability of *L. maculans* populations from different geographical locations (McGee & Petrie, 1978). Therefore, investigation on the effects of environmental factors on incubation period is needed in each region for a better understanding of the relationship among blackleg disease, canola and environment. see addendum 2 This study investigates the effects

of temperature, wetness duration, leaf position and cultivar on the incubation period of plants inoculated with ascospores of *L. maculans*, obtained from stubble in South Australia, in a controlled environment.

5.2 Materials and Methods

5.2.1 Experimental design

The experiment was carried out in a controlled-environment cabinet under fluorescent light with a 12 h photoperiod (described in section 2.2.1). The experiment, which was conducted three times, examined the effects of temperature (10, 15 & 20°C), wetness duration (16, 24, 48 & 72 h), cultivar (six cultivars differing in resistance to blackleg, see Table 2.1) and leaf position (leaves 1-3 with respect to the production of leaves on the seedling). As the incubation period of *L. maculans* at 5°C was long, the inoculated seedlings lost their first leaves and produced new leaves, so the 5°C treatment was discontinued. Three replicate pots were prepared per combination of temperature, wetness duration, canola cultivar and leaf position, with each pot containing one plant. Plastic trays (45 × 31 × 5 cm), containing 18 pots per wetness duration treatment, were placed in the growth cabinet (for each temperature treatment) and the pots for the six cultivars were arranged in a completely randomised design inside each tray. The same temperature was maintained until Phoma leaf spots appeared. Temperature inside the controlled-environment cabinet was monitored throughout the experiment using a Hastings data logger (Gemini Data Loggers (UK) Ltd). The incubation period was estimated as the time from inoculation until the first lesion was observed.

5.2.2 Plant material

Seeds of six canola cultivars, Q2, Karoo, Monty, ATR-Beacon, Ripper and Hyola 60 (see section 2.1.1), were sown in pots (7 × 8.5 × 5.5 cm) in UC soil (see section 2.1.2.1). Plants, thinned to one per pot, were grown initially in the glasshouse (see section 2.1.2.3). One day prior to inoculation, three replicate pots of each cultivar were transferred to each tray and placed in the controlled environment cabinet at the designated temperature. Immediately after inoculation, the trays were placed in the cabinet and kept filled with water (depth 1 cm) throughout the experiment. Inoculation of plants was performed approximately 2 weeks after sowing, at GS 1.4 (appendix 1). The first, second and third leaves of each plant were considered as three different leaf positions and inoculated as described below.

5.2.3 Inoculation

Pieces of tap root and stem base (*ca* 20-30 cm in length) of one-year-old canola stubble with blackleg cankers were obtained from a blackleg disease nursery in June 2003 (see section 2.1.3). Trays containing blackleg-affected stubble were prepared (as described in section 4.2.2.1) and placed in an incubator at 15°C under a 12 h photoperiod (see section 2.2.2). Stubble pieces were checked weekly and three pseudothecia were cut and examined microscopically until most pseudothecia had matured. Then the stubble pieces were left in trays lined with clean paper towel for a few hours to dry at room temperature (approximately 22°C), to prevent release of ascopores until they were required.

Stubble pieces with mature pseudothethecia were cut into segments (3-4 cm long), attached to the underside of Petri dish lids and moistened to stimulate ascospore release as described in section

4.2.2.1. After 2 h at 5°C, ascospores were released, then 1-2 ml sterile distilled water was added to the base of each Petri plate and the ascospores suspended using an artist's paint brush. The concentration was adjusted to 5×10^3 ascospores ml^{-1} with the aid of a haemocytometer.

After gently rubbing the adaxial surface of the leaf with a plastic eraser (Micador 110, Malaysia, Huang *et al.* (2001)) to improve adhesion of the spores, plants were sprayed with the ascospore suspension until run-off. Each tray containing 18 pots was placed in a plastic bag, which had been sprayed inside with water immediately after inoculation, to maintain humidity for the designated duration of wetness. Plants were given wetness periods of 16, 24, 48 and 72 h at each temperature from 5 to 20°C in the controlled environment cabinet (see section 2.2.1). After the designated wetness period, the plastic bags were removed and plants were placed in a plastic tent ($50 \times 105 \times 30$ cm) in the same controlled environment cabinet to provide conditions conducive to disease development. The plastic tent was used routinely to avoid drying out after preliminary experiments showed that lesions did not develop if plants were not kept in a humid atmosphere. The appearance of Phoma leaf spots was recorded macroscopically every day after inoculation. The number of days from inoculation to appearance of first leaf spots was recorded.

5.2.4 Effect of rubbing the leaf surface

To assess the effect of rubbing the leaf to partially remove the epicuticular wax on incubation period, an experiment was carried out with the same design described in section 5.2.1 except that leaves were not rubbed with a plastic eraser before inoculation. Three replicate pots were prepared per wetness duration, canola cultivar and leaf position treatment, with each pot

containing one plant as described in section 5.2.2, except that the experiment was conducted only at 15°C. This temperature was chosen as it was in the middle of the range examined.

5.2.5 Lesion development and recovery of *L. maculans* from inoculated plants

Following the recording of incubation period on the inoculated plants at each repetition of the experiment, recovery of *L. maculans* was attempted from Phoma leaf spots. Isolation of the pathogen was attempted from lesions (one lesion per leaf) on leaves 1-3 of three randomly selected plants per cultivar. Symptomatic leaves were excised and surface sterilized for 3 minutes in 0.5% sodium hypochlorite solution. The leaves were rinsed in sterile distilled water and dried in aseptic conditions in a laminar flow cabinet. Tissue from the margin of the lesion was transferred to PDA (appendix 2) amended with 30 mg l^{-1} streptomycin. Plates were incubated under fluorescent (Phillips TDL 36W/840, Thailand) and NUV (NEC T10 40W/FL40SBL, Japan) light at room temperature (approximately 22°C) for up to 2 weeks. Colonies grown from the tissues were identified microscopically based on the morphological characteristics.

5.2.6 Statistical analysis

The data from the three repetitions of the experiment conducted with rubbing leaves were subjected to ANOVA (see section 2.4) using factorial analysis, with temperatures, wetness duration, cultivar and leaf position as treatments (three replicates per repetition). Four-way interaction of temperatures, wetness duration, cultivar and leaf position was examined and significance was determined at the 5% level. If a factor or interaction was found to be statistically significant, LSD tests were conducted to determine which levels were significantly different.

Due to time constraints, the effect of rubbing the leaf with an eraser before inoculating the plant was not tested in a single experiment. However, the data from the experiment conducted at 15°C without rubbing leaves were compared with the data obtained for 15°C in the experiment with rubbing leaves, using factorial analysis in ANOVA (GenStat version 8.2, Lawes Agricultural Trust, Rothamsted Experimental Station, 2005). The conditions in both experiments were identical except for the leaf rubbing treatment (K. Dowling, pers. com., 2006).

5.3 Results

5.3.1 Effect of temperature, wetness duration, cultivar and leaf position

Ascospores of *L. maculans* infected leaves of all six canola cultivars and caused Phoma leaf spot lesions at temperatures from 10 to 20°C and leaf wetness durations from 16 to 72 h. The four-way interaction of temperature, wetness duration, cultivar and leaf position affected (LSD = 1.1; $P < 0.001$) the length of the incubation period on canola.

Overall at 10°C, the range of incubation period of *L. maculans* for the three leaf positions on the six cultivars was 8.8-18.3 days over 16-72 h wetness (Figure 5.1). At 10°C, the incubation period was shorter ($P < 0.05$) following 24 h wetness (on leaves 2 and 3) and following 16, 48 and 72 h wetness (on all three leaf positions) on Hyola 60 than on the other cultivars. The shortest incubation period at 10°C was observed on leaf 1 of Hyola 60 (8.8 days) following 16 h wetness. The longest incubation periods following exposure to 16, 24, 48 and 72 h wetness were observed on leaf 3 of Karoo (18.4 days), Ripper (16.7 days), and Monty (16.1 days), and leaf 1 of Ripper (15.7 days), respectively. The incubation period on leaf 3 of Karoo following 16 h wetness at

10°C was greater ($P < 0.05$) than on any leaf position of any cultivars. The largest differences in incubation period between leaf positions of any one cultivar at 10°C were mostly observed following 16 h wetness duration, with disease taking longer ($P < 0.05$) to appear on leaf 3 than on leaf 1 of all cultivars except Beacon.

At 15°C, the range of incubation period of *L. maculans* on the three leaf positions of the six cultivars was 7.4-10.1 days over 16-72 h wetness (Figure 5.2). Following 16 h wetness, incubation period was longer ($P < 0.05$) on leaf 3 than leaf 1 of Karoo and on leaf 2 and 3 than leaf 1 of Q2. No significant difference in incubation period was observed among cultivars or leaves at 15°C with 24 h and 72 h wetness except Monty, on which incubation period was longer ($P < 0.05$) on leaf 3 than leaf 1 and 2 following 72 h wetness. With 48 h wetness, incubation period was longer ($P < 0.05$) on leaf 3 of Q2, Monty and Ripper than leaf 1 or 2. The minimum and maximum mean incubation periods of *L. maculans* at 15°C were observed on leaf 2 (7.4 days with 16 h wetness) and leaf 3 (10.1 days with 48 h wetness) of Ripper, respectively.

The range of incubation period of *L. maculans* for the three leaf positions on the six cultivars was 3.3-7.6 days over 16-72 h wetness at 20°C (Figure 5.3). At 20°C, the mean incubation period was generally longer following 16 h of leaf wetness than other wetness treatments for all cultivars tested. Incubation period was 2.2-3.2 days longer ($P < 0.05$) on leaf 3 of all cultivars following 16 h of incubation compared with 24 h at 20°C. Cultivar did not affect mean incubation period on each leaf position at 20°C with 16 h wetness and incubation time was longer ($P < 0.05$) on leaf 3 than leaf 1 only for Monty and Q2. With 24-72 h wetness at 20°C, cultivars and leaves showed slight, but significant differences in incubation period. The shortest incubation period was on leaf 1 or leaf 2 of Hyola 60 (3.3-4.9 days) with 24-72 h wetness. The longest incubation periods at

20°C following 24, 48 and 72 h wetness durations were on leaf 1 of Monty (6.1 days), leaf 1 of Ripper (6.4 days) and leaf 2 of Q2 (6.8 days), respectively.

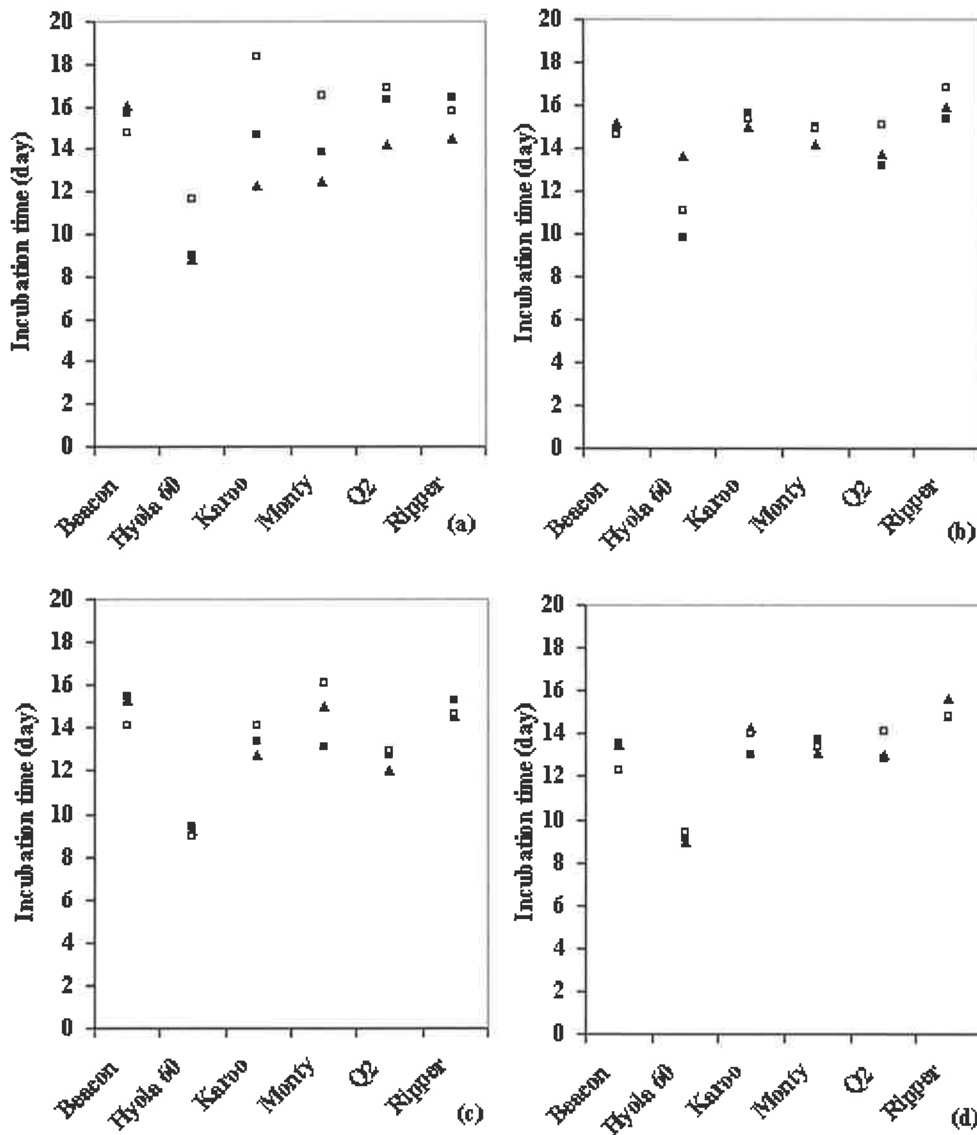


Figure 5.1. Mean incubation period (the time from inoculation to the appearance of the first Phoma leaf spot lesions) for the three repetitions of the experiment on first (▲), second (■) and third (□) leaves of six canola cultivars inoculated with ascospores of *Leptosphaeria maculans* after 16 (a), 24 (b), 48 (c) and 72 h (d) wetness duration at 10°C; LSD = 1.1; P < 0.05.

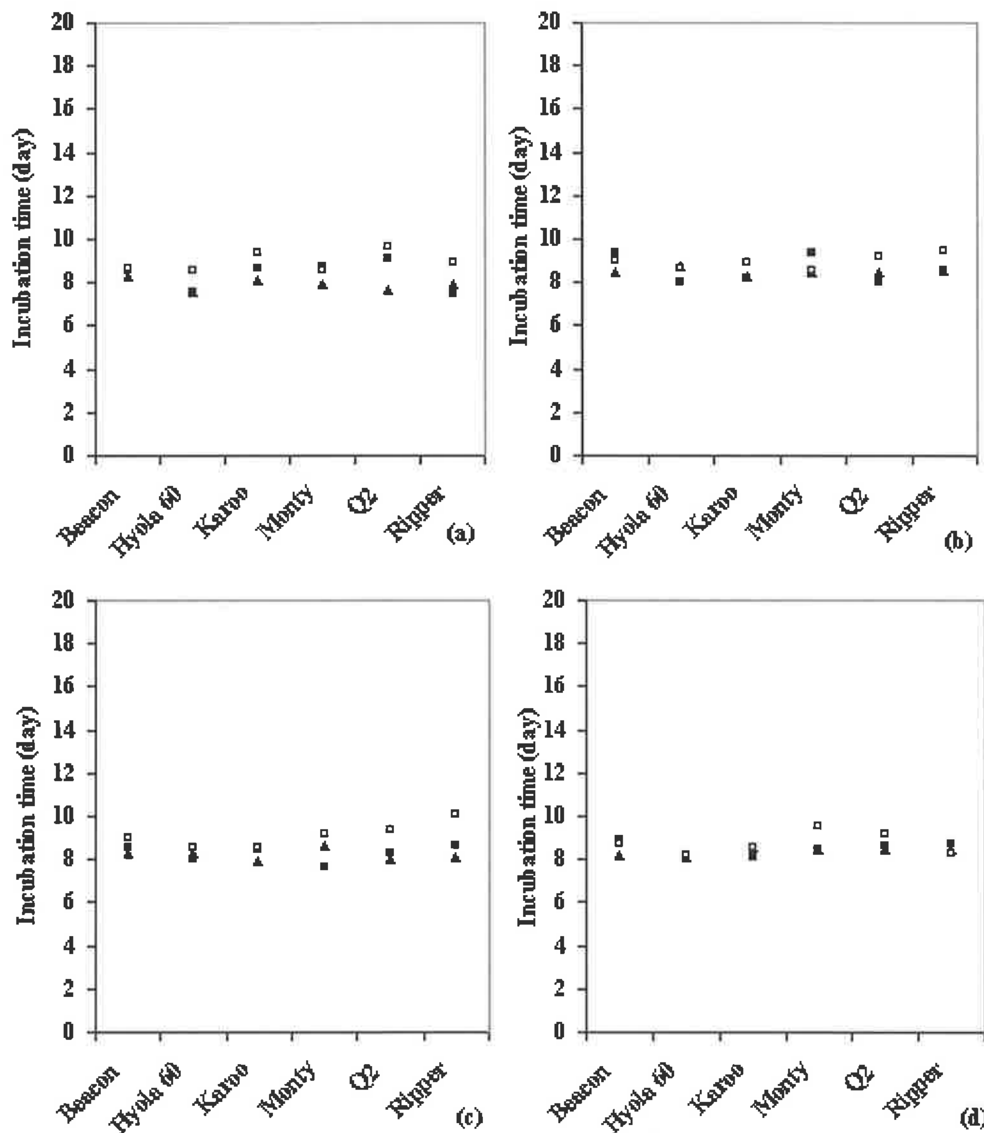


Figure 5.2. Mean incubation period (the time from inoculation to the appearance of the first Phoma leaf spot lesions) for the three repetitions of the experiment on first (▲), second (■) and third (□) leaves of six canola cultivars inoculated with ascospores of *Leptosphaeria maculans* after 16 (a), 24 (b), 48 (c) and 72 h (d) wetness duration at 15°C; LSD = 1.1; $P < 0.05$.

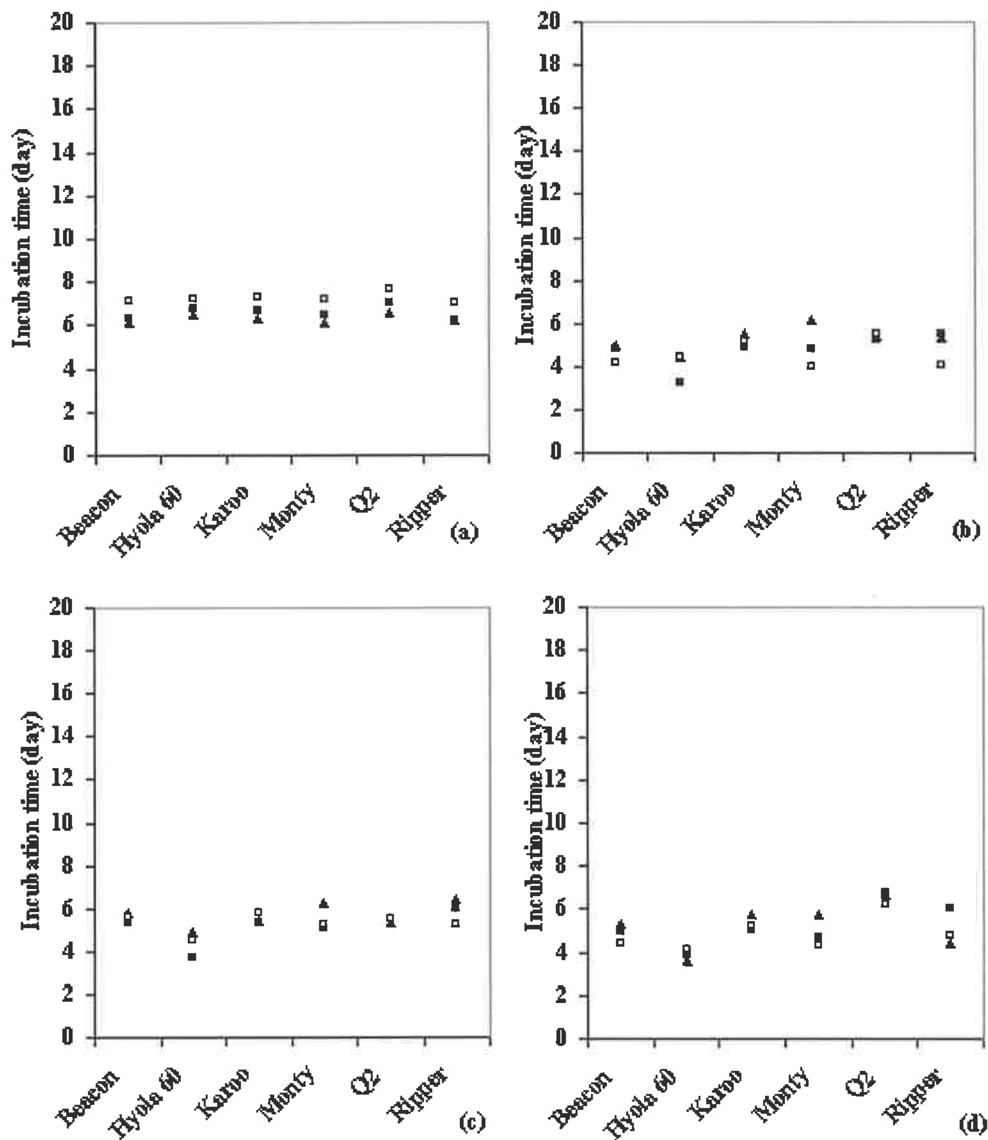


Figure 5.3. Mean incubation period (the time from inoculation to the appearance of the first Phoma leaf spot lesions) for the three repetitions of the experiment on first (▲), second (■) and third (□) leaves of six canola cultivars inoculated with ascospores of *Leptosphaeria maculans* after 16 (a), 24 (b), 48 (c) and 72 h (d) wetness duration at 20°C; LSD = 1.1; $P < 0.05$.

Overall, the mean incubation period was significantly longer ($P < 0.05$) for each leaf position for all cultivars with each wetness duration for plants incubated at 10°C than at 15°C, except for Hyola 60, leaf 1 and leaf 3 following 48 h wetness and leaf 1 and leaf 2 following 72 h wetness (Figures 5.1 and 5.2). The mean incubation period of *L. maculans* for each leaf position of all cultivars was significantly shorter ($P < 0.05$) for plants kept at 20°C than at 15°C, except for leaf 2 of Hyola 60 with 16 h wetness (Figures 5.2 and 5.3). The duration of the incubation period was more variable at 10°C than at 15 and 20°C (Figures 5.1, 5.2 and 5.3).

For ease of comparison among the three temperatures tested (analysed as the four-way interaction), the general trend for mean incubation period with respect to leaf position is illustrated for cv. Monty (representative cultivar) in Figure 5.4. The incubation period was significantly longer ($P < 0.05$) on leaves 1, 2 and 3 of Monty at 10°C than 20°C. The difference in the duration of incubation period generally was more marked when comparing 10 with 15°C than 15 with 20°C (Figure 5.4).

5.3.2 Effect of rubbing the leaf surface

The four-way interaction of rubbing the leaf (with an eraser before inoculation of canola plants), wetness duration, cultivar and leaf position did not affect ($P = 0.9$) the incubation period at 15°C. However, the three-way interaction of rubbing factor, cultivar and leaf position affected ($P = 0.02$) the incubation period (Table 5.1). The incubation period at leaf 1 of Q2, at leaf 2 of Q2 and ATR-Beacon, and at leaf 3 of Q2, Karoo and Ripper, was longer ($P < 0.05$) on non-rubbed than rubbed leaves.

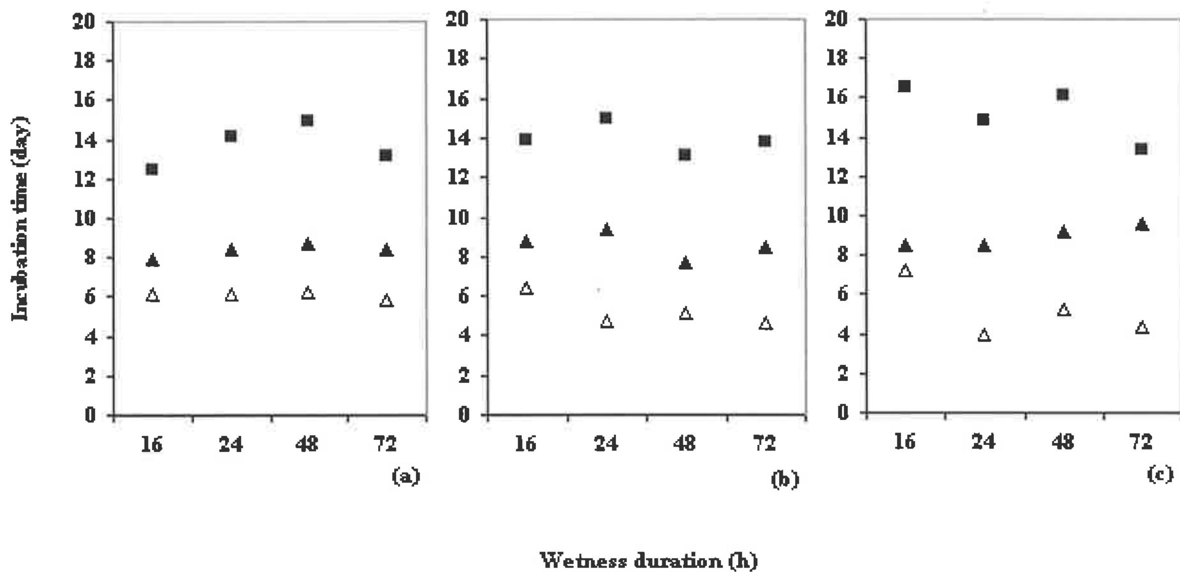


Figure 5.4. Mean incubation period of *Leptosphaeria maculans* for the three repetitions of the experiment at three leaf positions, (a) leaf 1, (b) leaf 2 and (c) leaf 3, of canola cv. Monty maintained at 10°C (■), 15°C (▲) and 20°C (△) following 16-72 h wetness duration; LSD = 1.1; P < 0.05.

5.3.3 Lesion development and recovery of *L. maculans* from inoculated plants

Lesions generally began as pinpoints and expanded to light grey areas with pycnidia, except for those on Hyola 60, where some lesions remained as small, black spots and others expanded more slowly than on other cultivars. The pathogen produced larger pale grey lesions containing more pycnidia on the leaves of the susceptible cultivars than the resistant cultivars, excepting cv. ATR-Beacon (Figure 5.5). The lesions enlarged faster on leaves of susceptible cultivars and ATR-Beacon than of resistant cultivars. Colonies of *L. maculans* developed from all tissue samples from lesions on leaves of the 18 inoculated plants when cultured on PDA.

Table 5.1. Mean incubation period (days) for leaves at three positions on six canola cultivars at 15°C following 16-72 h wetness duration with and without rubbing leaves before inoculation with *Leptosphaeria maculans* from the three repetitions of the experiment.

Cultivar	Leaf position					
	1		2		3	
	R	N	R	N	R	N
Hyola 60	8.2[1]	7.9	7.9	7.4	8.5	8.8
Ripper	8.2	8.1	8.4	8.1	9.2	10
ATR-Beacon	8.3	8.4	8.8	9.4	8.9	8.9
Monty	8.4	8.6	8.6	9	9	9.5
Karoo	8.2	8.3	8.4	8.4	8.8	9.5
Q2	8.1	8.7	8.5	9.1	9.4	10

R = leaves rubbed gently with a plastic eraser before inoculation; N = leaves not rubbed before inoculation.

[1] LSD = 0.6; $P < 0.05$.

5.4 Discussion

Ascospores of *L. maculans* infected six canola cultivars that differed in blackleg-resistance rating over 10-20°C and leaf wetness durations of 16-72 h. This supports the findings of Biddulph *et al.* (1999b) and Toscano-Underwood *et al.* (2001) that ascospores of *L. maculans* can infect canola over a range of temperatures and wetness durations. These results also provide additional information on the effects of environmental factors, cultivar and leaf age on incubation period.

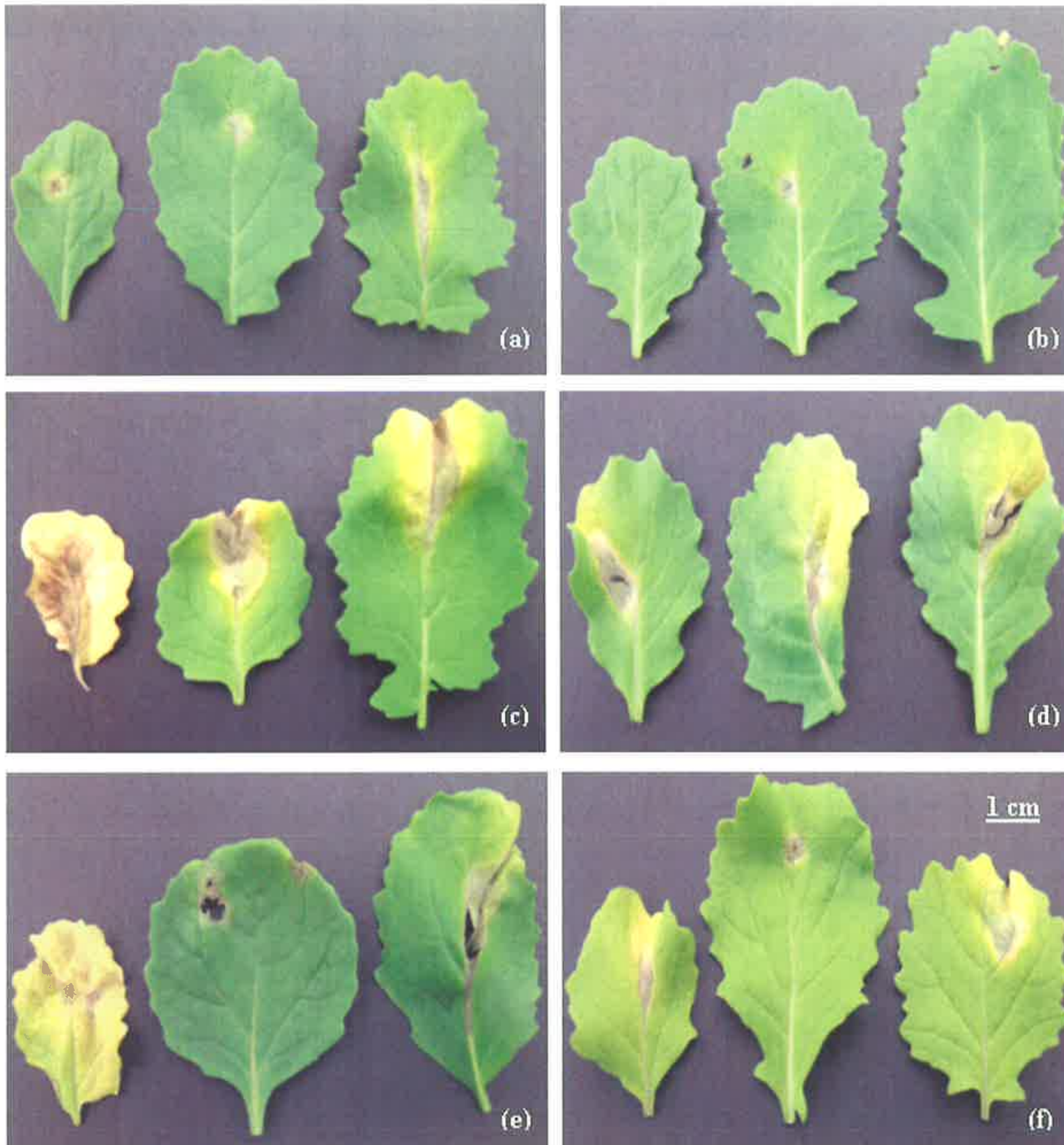


Figure 5.5. Typical Phoma leaf spot lesions formed on leaves at three positions (1-3 from left to right) of six cultivars of canola inoculated with *Leptosphaeria maculans* ascospores 8 days after inoculation and incubation at 20°C; (a) ATR-Beacon, (b) Hyola 60, (c) Karoo, (d) Monty, (e) Q2, (f) Ripper.

The finding that incubation period of *L. maculans* on canola decreased with increasing temperature from 10 to 20°C on most cultivars supports earlier findings (Biddulph *et al.*, 1999b; Toscano-Underwood *et al.*, 2001). Previous studies showed that 10°C and wetness duration of 16 h are suboptimal conditions for infection (Biddulph *et al.*, 1999b; Toscano-Underwood *et al.*, 2001). In the present study, significant differences were observed among the canola cultivars and three leaf positions following a range of wetness periods at 10°C. In addition, effect of incubation period at the three leaf positions of all cultivars was most variable at 10°C following 16 h wetness duration. Plants incubated at 10°C with wetness period of 16 h developed leaf lesions earlier on older (first) leaves than on younger (second and third) leaves. The combination of suboptimal conditions at 10°C, 16 h wetness and youngest leaf explains why the longest incubation period, 18.4 days, was recorded on leaf 3 at 10°C with 16 h wetness. Although Biddulph *et al.* (1999b) reported that leaf wetness duration affected the length of the incubation period only at suboptimal temperatures, in the present study, wetness duration affected the incubation period at both 20°C (optimum) and 10°C (suboptimum) temperatures. For example, the incubation period following 16 h wetness duration at 20°C was longer than at following other wetness durations. The differences between findings may be due to differences in genotypes of the host and the pathogen, and require further investigation. See Addendum 3.

The incubation period of *L. maculans* on most cultivars for each leaf position and at each wetness period decreased more when temperature increased from 10°C to 15°C than from 15°C to 20°C, suggesting that 20°C was the optimum of the range tested here, which supports earlier findings (Biddulph *et al.*, 1999b; Toscano-Underwood *et al.*, 2001). The only exception was observed on the leaves of Hyola 60, for which the mean incubation period at each wetness period showed slight or no decrease when temperature increased from 10°C to 15°C.

Cultivar affected the length of the incubation period at 10-20°C. However, the effect of cultivar on incubation period was not related to blackleg-resistance rating. It was expected that the incubation period would be longer on resistant cultivars than susceptible ones, but results showed various and inconsistent reactions of the six cultivars at different temperatures following different wetness durations. This supports recent findings that phenotypic expression of resistance gene, *Rlm6*, in canola leaves (inoculated with ascospores or pycnidiospores) and subsequent development of stem cankers was affected by temperature from 5 to 25°C and wetness duration for 12-72 h, and number of lesions generally increased with increasing temperature and wetness duration (Huang *et al.*, 2006). Furthermore, findings may indicate a lack of resistance at the surface of leaves of resistant cultivars. As the leaf surface is the infection court, resistance at this stage may prevent or reduce infection of the plant and, consequently, reduce canker and yield loss. Therefore, the selection of material with leaf resistance to *L. maculans* should be considered in breeding programs to improve crop yield.

Likewise, leaf position affected the incubation period following 16h wetness at 10-20°C, suggesting that leaf age may be important in the infection of canola leaves by *L. maculans* ascospores. In addition, leaf position appeared to be more important than wetness duration and cultivar, within the range of temperature, wetness duration, cultivar and leaf position tested. However, the effect of leaf age on incubation period is likely to be less important than temperature, as changes in temperature resulted in larger differences in incubation period. Furthermore, incubation period was longer on younger leaves than older leaves at 10°C and 20°C following 16 h wetness and also at 15°C, regardless of wetness duration. This suggests that incubation period decreases with increasing leaf age, regardless of blackleg-resistance rating of cultivars. The results differed from earlier

findings in which leaf age did not affect the incubation period on plants inoculated with pycnidiospores of *L. maculans* (Vanniasingham & Gilligan, 1989). It is possible that the type of inoculum used affects the length of the incubation period. Whereas Poisson & Pérès (1999a) reported longer incubation period at the 2-leaf stage than 6-leaf stage for all cultivars and on the resistant cultivar than susceptible cultivars, incubation period did not depend on blackleg-resistance rating of cultivars and was generally longer on first leaf than on second or third leaf in the present research. Differences in methodology, the host plant and the pathogen genotypes might have caused the differences in the findings.

Gently rubbing the leaves in order to improve adhesion of ascospores may have resulted in partial disruption of the wax layer and leaf exudates, and had a slight, but significant, effect on incubation period in comparison with non-rubbed leaves. Incubation period was shorter on rubbed than on non-rubbed leaves of Q2 at 15°C, regardless of wetness duration. As incubation period is the time from inoculation to the appearance of leaf spots, it includes germination of spores, penetration into leaf tissues, growth and symptom development. The effect of rubbing leaves suggests that partially removing leaf surface exudates and wax may facilitate germination on or penetration of the leaf by ascospores of *L. maculans*, as was shown by Conn & Tewari (1989) for conidia of *Alternaria brassicae* (see section 4.1). However, further research is required to confirm the finding and clarify the role of leaf surface wax or exudates on infection, and to determine if the content and composition of wax of plants grown outdoors and in a controlled environment differ.

In general, the type of lesions formed on leaves inoculated with ascospores was similar among cultivars, except Hyola 60. Although the leaf lesions on Hyola 60 often appeared earlier than other cultivars, those remained as black points or developed more slowly than those on the leaves of other cultivars tested. This may reflect a hypersensitive response in the leaf of Hyola 60, which

is highly resistant to some genotypes of *L. maculans*. Similarly, Hua *et al.* (2004) reported the rapid development of small, dark lesions around the penetration site on cotyledons of resistant cultivar, Surpass 400, inoculated with either ascospores or pycnidiospores. However, in the present study, that resistance mechanism did not inhibit the pathogen at the site of infection on Hyola 60 and allowed the restricted or slow development of leaf lesions and formation of pycnidia. [See Addendum 4.] Previously, Potter *et al.* (2003) reported that only 20% of plants cv. Hyola 60 displayed leaf spots compared to 100% of plants of all other cultivars inoculated with pycnidiospores in the field. Germination and hyphal growth from pycnidiospores were severely restricted on the leaf surface of Hyola 60, suggesting the presence of an inhibitory substance (Potter *et al.*, 2003). Such differences between cultivars could be investigated in future research to detect any inhibitory substances, as a means of producing cultivars resistant to leaf infection.

This study provides new knowledge about the influence of cultivar and leaf position on the incubation period of *L. maculans* on canola and provides information towards a better understanding of the factors affecting the disease. In addition to information about infection, knowledge of survival of the pathogen inoculum on stubble is required for a better understanding of the disease and for optimizing control strategies.

Chapter 6. Survival of *L. maculans* and associated mycobiota on buried canola stubble and changes in soil microbiota

6.1 Introduction

Epidemics are initiated by ascospores released from pseudothecia that develop on stubble left in the field after harvest (McGee & Petrie, 1979). In Canada, buried canola stubble underwent 1.6 to 2 times more decomposition than stubble on the soil surface, with an average of 70% more weight loss from stems than from roots and a small effect of cultivar on decomposition of stubble (Blenis *et al.*, 1999). In south-eastern Australia, only 10% of canola stubble remains on the soil surface 12 months after harvest and ascospore discharge decreases with increasing stubble age (Marcroft *et al.*, 2003a) and with the use of resistant cultivars (Marcroft *et al.*, 2004). In contrast, in Alberta, Canada, it was reported that stubble left on the soil surface remained intact for 36 months, however, the weight of stubble decreased by 40-60% within a year of burial (Kharbanda & Ostashewski, 1997). A DNA-based assay for *L. maculans* detected the pathogen mainly in the upper 5 cm of the soil profile and *L. maculans* decreased to negligible amounts over 3 years or more after cropping (Sosnowski *et al.*, 2001a; Sosnowski *et al.*, 2006). Currently, in south-eastern Australia, canola growers are encouraged to follow a 4-year rotation with other crops to allow sufficient time for blackleg-affected stubble to decay. However, the high profitability of this crop and benefits of rotations with cereal crops have significantly increased the area sown to canola and the time between successive canola crops in the same field has decreased. Consequently, the large amount of canola stubble produces more ascospore showers, thereby increasing the disease pressure (Marcroft *et al.*, 2003a).

The influence of burying infested canola stubble on development of pseudothecia was studied in Alberta, Canada (Kharbanda & Ostashewski, 1997). Pseudothecia formed on buried stubble of highly susceptible cultivars in one month, whereas stubble of moderately resistant cultivars bore pseudothecia only after 10 months of burial. Although pseudothecia developed on standing stubble about a year after harvest in Canada, ascospore release from pseudothecia on standing stubble of the same year crop has been reported in the US (Kharbanda & Ostashewski, 1997). Although this comparison suggests that the role of environment and population structure of the pathogen may be important, the effect of burial on differential development of pseudothecia on canola stubble is little understood.

In the UK, mycelium of *L. maculans* survived on stubble placed on the surface of sand or buried in sand for 12 months and produced pseudothecia and viable ascospores (Huang *et al.*, 2003). However, the effect of soil factors on density of pseudothecia or pathogenicity of ascospores was not considered in the British study. It was reported that the isolation frequency of *L. maculans* decreased from 85% before burial in July (summer) to 15.4% in September and 3.4% in December in the United States (Baird *et al.*, 1999). Whereas the frequency of isolation of most stubble-associated fungi decreased over a year of burial, the isolation frequency of *Trichoderma* spp. increased to 27% over the same period. Depth of burial (0, 7.6 and 25.4 cm) or location (two sites, with Lloyd clay loam and Davidson loam soil types) did not affect the isolation frequency of *L. maculans*, however, the fungus survived longer on intact stubble left on the soil surface than on stubble buried in soil. In Canada, none of 30 fungal species (39 isolates) from wood or eight species from canola stubble (*Cyathus olla*, *Coprinus* sp. and six unidentified isolates) were effective in eliminating *L. maculans* from stubble, nor did they cause significant decomposition of non-sterile stubble either under laboratory conditions or in the field (Blenis & Chow, 2005).

Management of blackleg disease through stubble decomposition needs further effort to identify effective decomposers and antagonists of *L. maculans*.

Baird *et al.* (1999) suggested that the rate of biodegradation of stubble is dependent on a number of environmental factors, such as temperature and moisture, which can influence the activity of soil microorganisms. Changes in the mycobiota induced by various crops and crop rotations, and knowledge of the factors affecting these changes, are important to the understanding of the relationship of crop sequences to disease occurrence in the field. Also, such changes have been reported when crop residue has been added to soil, e.g. the addition of oat straw to soil decreased isolation of *Fusarium* spp. (Menon & Williams, 1957). Although fluctuations have been reported in populations of bacteria and fungi due to changes in soil temperature and moisture, Menon and Williams (1957) observed no qualitative or quantitative differences in mycobiota of soils maintained for 5 weeks at 10, 20 or 30°C. Likewise, there was no difference in the isolation frequency of most fungi from soils with different moisture content. However, total numbers of fungi were greater at low than at high moisture contents due to smaller populations of *Botrytis* spp. and *Penicillium* spp. in moist soil. Soil pH did not influence mycobiota changes. Menon and Williams (1957) pointed out that the distribution of mycobiota depends on the nature and amount of utilizable substrates in soil, and the crop or crop debris affects the mycobiota directly. The influence of canola stubble on microorganism populations in soil is not well understood.

Furthermore, the effect of environment and soil characteristics on survival of *L. maculans* is not clearly understood. Therefore, this study examined the effect of burying canola stubble and the impact of stubble mycobiota on survival, pseudothecium formation and pathogenicity of *L. maculans*, and changes in soil microbiota over time in two soil types in South Australia.

6.2 Materials and Methods

6.2.1 Burial of canola stubble in sand and field soil

Field soil and sand were collected in February 2004 and analyzed to determine pH, texture and electrical conductivity (EC). Soil was collected from the Charlick experiment station, South Australia, 50 km south of Adelaide, from a field with a cropping history of canola-wheat-pulse-wheat-barley from 1999 to 2003. Sand (Tailem Bend Sandy Loam, Sloan's Sands Pty Ltd, South Australia) was used for comparison with the field soil. Forty-eight black pots (20 cm diameter) were filled with soil or sand.

The EC and pH of the soil and sand were determined using a solution prepared from each soil type, using a conductivity meter and a pH meter, respectively (Rayment & Higginson, 1992). To make 1: 5 solutions, 40 ml of RO water was added to 8 g of air dried (2 mm sieved) sand or soil and placed on a shaker for half an hour. After 2 h at room temperature, EC (Cl^- concentration) and then pH values of each soil solution were measured. The texture of sand and field soil was determined using the hydrometer method (Madison *et al.*, 1986) as follows: 50 ml of 10% Calgon (sodium metahexaphosphate), 5 ml of 0.6 m NaOH and then 500 ml RO water were added to 40 g of oven dried (65°C) sand or soil. The solutions were vigorously shaken and left on a shaker for 24 h. RO water was added to both solutions to make 1 L and mixed vigorously. Hydrometer measurements were taken at 5 minutes and 5 h and the formulae below were used to calculate percent clay, silt and sand. Then Marshall's diagram, relating mechanical analysis percentages, was used to determine the texture of the sand and soil (Madison *et al.*, 1986).

X = Hydrometer reading in the solution (Calgon, NaOH and water) before adding the soil

5 minutes reading - X = Silt + Clay

5 h reading - X = Clay

%Clay = Clay/ The soil weight (40 g)

%Silt = Silt/ The soil weight (40 g)

%Sand = 100 – (%Clay + %Silt)

Canola stubble, which consisted of 40-80 cm long stem and root pieces, was obtained from a blackleg disease nursery in January 2004 (see section 2.1.3). Basal parts of the stems (from crown to 30 cm of stem) were cut into 10-cm pieces. Ninety-six lots, comprising 20 pieces each, were individually weighed (8.7-29 g) and placed in 20 cm^2 plastic mesh bags (mesh size 1 mm^2). These were buried 5-10 cm deep in separate pots filled with sand or field soil. All 96 pots, each containing one bag of stubble, were placed on sand in an external environment at the Waite Campus, South Australia in February 2004. The pots were labelled and arranged randomly on the sand (Figure 6.1a, b).

6.2.2 *L. maculans* and associated mycobiota on buried canola stubble

In January 2004, prior to placing the canola stubble in the bags, 10 pieces (10 cm long) were randomly selected to examine the indigenous mycobiota, using the isolation method described below. Subsequently, four replicate bags from each soil type were destructively sampled and stubble assayed for survival of *L. maculans* each month over the 13-month period. In addition, the appearance of the stubble with regard to decomposition, such as integrity of the epidermal

layer, stem pith and breakage, was recorded. No stubble sample was assessed in July 2004, due to unforeseen circumstances. On retrieval, the stubble from each bag was immediately rinsed in running tap water to remove excess soil, dried for 48 h at room temperature and then weighed. Ten stubble pieces were randomly selected from each bag, three 1 cm sections were excised, one from each end and the middle, then surface sterilized in 0.5% sodium hypochlorite solution for 3 minutes initially. The duration of surface sterilization was reduced to 1 minute after 7 months, due to the decomposition of stubble. The stubble was rinsed in sterile distilled water and left on sterile filter paper for 2 h to dry, then plated onto dilute V8 juice agar (DV8A, described in appendix 2) amended with streptomycin (Erwin *et al.*, 1987). The cultures (three pieces in each 9 cm Petri dish) were incubated on a bench (68 × 138 cm) under fluorescent light (Phillips TDL 36W/840, Thailand) and black light (NEC T10 40W/FL40SBL, Japan) at room temperature (approximately 22°C) up to 10 days. Fungal colonies were transferred to PDA amended with 30 mg l^{-1} streptomycin.

All fungi isolated were identified and the isolation frequency of each was recorded for each stubble sample. The frequency of isolation for each fungus was calculated as the total number of isolations from all 30 stubble pieces from each bag and expressed as a percentage, such that, if a particular fungus was isolated from all 30 pieces, then isolation frequency was 100%. Both macroscopic and microscopic cultural characteristics were used to identify the fungi, mostly to genus, using general identification keys (Barnett & Hunter, 1998; Domsch *et al.*, 1980; Ellis, 1971; Ellis, 1976). Single spores of cultures identified as *Fusarium* spp. were transferred to carnation leaf agar and identified to species level (Nelson *et al.*, 1983). Fungi that were isolated frequently were identified to species. Isolates representative of the predominant genera, and unknown species, were sent to Dr Michael Priest, New South Wales Agriculture, for identification and deposition in

the Plant Pathology Herbarium (DAR), Orange Agricultural Institute, New South Wales, Australia.

Figure 6.1. Ninety-six pots containing bags of stubble buried in sand or field soil with labels in the centre, placed on sand in an external environment close to the weather station (arrow) at the Waite Campus, South Australia in February 2004.

6.2.3 Effect of environmental factors and soil temperature

The temperature of the soil inside one pot of each soil type was recorded using Hastings Data Loggers (Gemini Data Loggers (UK) Ltd), from March 2004 to February 2005. The data were recorded hourly then converted to mean daily then to mean monthly temperature for sand and field soil. Air temperature and rainfall were recorded daily by a weather station (Model T metstation, Western Electronic Design, Loxton, South Australia) from March 2004 to February

2005. The mean monthly temperature and rainfall were obtained from mean daily records by the weather station.

6.2.4 Pseudothecium formation on buried canola stubble

6.2.4.1 Effect of storage

Study of pseudothecium formation on canola stubble buried in sand and field soil, in February 2004, and pathogenicity of the resulting ascospores on canola plants began in September 2004. As stubble retrieved prior to this time was stored at room temperature, an experiment was conducted to investigate the effect of the storage on pseudothecium density on the stubble. Ten pieces of stubble from four bags, sampled in September 2004 from each soil type, were randomly selected. After moist incubation at 15°C to produce mature pseudothecia, the density of pseudothecia on each piece of stubble was estimated (as described in section 3.2.1). The remaining stubble was then stored at room temperature until February 2005 (5 months storage) and the density of pseudothecia was determined as above.

6.2.4.2 Effect of burial

Each month, 15 pieces of stubble were removed at random from those pieces remaining in the four bags (stored at room temperature after removal of stubble from each bag for isolation of fungi) from each type of soil. The presence of pycnidia or pseudothecia on the stubble was recorded. The stubble pieces from each soil type were then soaked separately in sterile distilled water for 12 h, and then five pieces from each soil type were placed in each of three replicate trays to induce pseudothecium development, as described in section 3.2.1, and then placed in an

incubator at 15°C under a 12 h photoperiod (see section 2.2.2). Maturation of pseudothecia was assessed (see section 3.2.1) every 5 days. At the last assessment, the density of pseudothecia on each piece was assessed (see section 3.2.1) and calculated as the mean from the five stem pieces in each tray.

6.2.5 Pathogenicity of *L. maculans*

Each month, seeds of cv. Karoo (see section 2.1.1), were sown in pots (7 × 8.5 × 5.5 cm) containing UC soil (see section 2.1.2.1). Plants were grown in a greenhouse (see section 2.1.2.3) and thinned to one plant per pot. Five replicate pots were placed in a plastic tray (31 × 45 × 5 cm) for each soil type and watered as required. Plants at growth stage 1,4 (appendix 1) were used in a bioassay to assess the pathogenicity of ascospores.

Ten pieces of canola stubble (10 cm long) from each soil type, with mature pseudothecia (obtained from the experiment described in section 6.2.4.2), were sectioned into three pieces (3-4 cm long) and used to prepare suspensions of 5×10^3 ascospores ml^{-1} (see section 4.2.2.1). For each soil type, five seedlings at growth stage 1,4 were sprayed with the ascospore suspension until leaf surfaces were uniformly covered with droplets. The inoculated plants were covered with plastic bags, which were sprayed inside with water and then placed in a controlled environment cabinet at 20°C in 12 h photoperiod (see section 2.2.1). After 72 h, plastic bags were removed and plants were moved to a plastic tent (50 × 105 × 30 cm) to maintain a humid environment. The appearance of Phoma leaf spots was recorded 7 days after inoculation.

6.2.6 Soil microbiota

Each month over the 13-month period, four 2.5 g samples from the soil surrounding the stubble bag from four separate pots of the field soil were collected. The samples were pooled and mixed thoroughly before use. The 10 g sample was diluted serially in Ringer's solution (McLean & Ivimey-Cook, 1941) and 1 ml aliquots of the 10^{-4} dilution, for fungi, and 10^{-6} dilution, for bacteria, were plated on five replicate Petri dishes each of peptone-rose bengal agar (PRA, described in appendix 2) and crystal violet agar (CVA, described in appendix 2), respectively (Bakerspigel & Miller, 1953; Martin, 1950). Aliquots of the 10^{-6} dilution were also plated on four replicate Petri dishes of soil-extract agar (SEA, described in appendix 2) to estimate the total microbiota in the soil samples (James, 1958). After 10 days of incubation at room temperature with a 12 h photoperiod (described in section 6.2.2), the number of colony forming units (CFU) per gram soil on each medium was determined.

Fungal colonies on PRA were transferred to PDA amended with 30 mg l^{-1} streptomycin and incubated to produce spores. All fungi were then identified to genus level based on macroscopic and microscopic cultural characteristics (described in section 6.2.2) and the CFU per gram soil for each fungus was estimated. Bacterial colonies isolated on CVA were transferred to nutrient agar (NA, described in appendix 2) and incubated for 5 days. Gram categories were determined by the KOH test, as follows. A loopful of growth from a 7-day-old colony of the organism on NA was emulsified on a glass slide in a drop of 3% KOH. The suspension was stirred continuously for 60 s after which the loop was gently pulled from the suspension. The test bacterium was considered Gram-positive if the suspension appeared stringy within the first 30 s of mixing the

bacteria in KOH solution (Gregerson, 1978). The CFU per gram soil for each colony type of bacteria isolated on CVA was also recorded.

6.2.7 Statistical analysis

Data for the daily average temperature of the two soils were subjected to regression analysis using GenStat version 6.1 (Lawes Agricultural Trust, Rothamsted Experimental Station, 2002) to compare temperature over time. The difference in stubble weight before and after burial of the bags in sand and field soil was calculated and converted to a percentage, then the data for percent weight reduction of stubble over 13 months were subjected to analysis of variance (ANOVA) using GenStat. The rate of percent decrease of stubble weight was calculated as follows:

$$\text{Rate} = \frac{\text{Percent stubble weight decrease}}{\text{Number of month of burial}}$$

The data for frequency of isolation of each fungus from canola stubble buried in the sand or field soil over 13 months were subjected to ANOVA. To determine the trend in isolation frequencies across months, linear and quadratic analyses were included (K. Dowling, pers. com., 2005). The relationship between weather records, temperature and rainfall, and fluctuations in the isolation frequency of those fungi that showed significant difference over the duration of the experiment was investigated using regression analysis (M. Lorimer, pers. com., 2005).

To evaluate the effect of storage on pseudothecium formation, the mean density of pseudothecia on stubble from sand and field soil retrieved in September 2004 was compared with that on

stubble from the same retrieval time, stored until February 2005, using ANOVA. To evaluate the effects of soil type and time (duration of burial) on formation of pseudothecia, data for the time to reach each stage of maturity and data for the density of pseudothecia on the stubble buried in the sand or field soil over 13 months were subjected to ANOVA. Data for the number of CFU of fungal genera isolated on PRA, bacterial colony types isolated on CVA, fungi and bacteria isolated on SEA in the field soil over 13 months were subjected to ANOVA (K. Dowling, pers. com., 2005).

6.3 Results

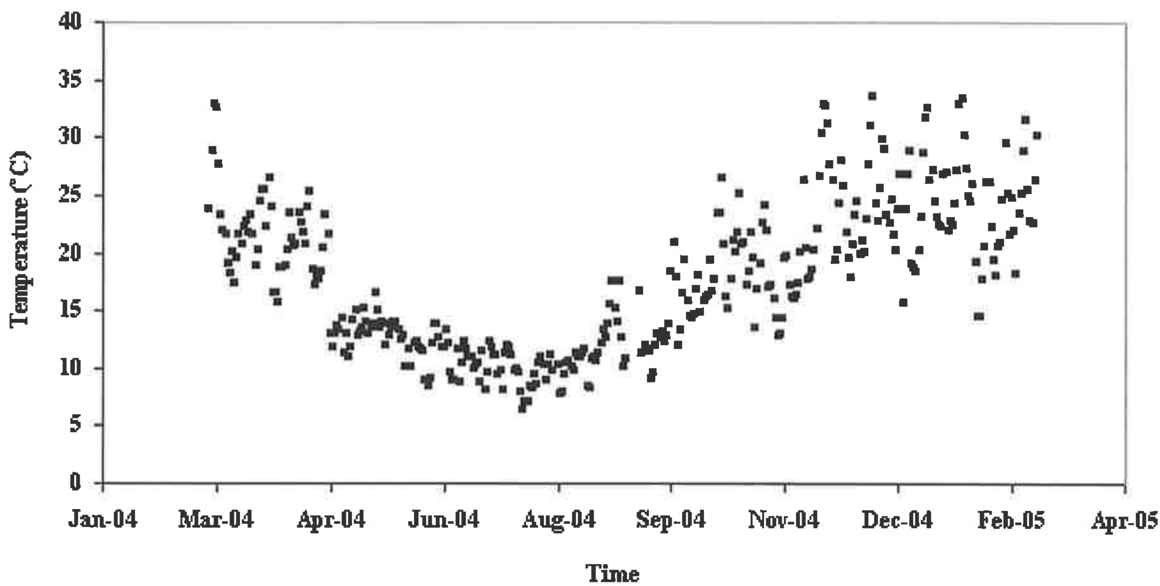
6.3.1 Burial of canola stubble in sand and field soil

The field soil was categorized as having a clay-loam texture, pH 7.9 and electrical conductivity (EC) of 102.4 mg l^{-1}. The pH of the sand was 7.3 and the EC was 44.8 mg l^{-1}. There was no significant difference in daily average temperature between the two soils over the 12 months (Figure 6.2). In sand, the maximum daily temperature of 34.1°C and the minimum of 6.6°C occurred in December and in July, respectively. In field soil, the maximum daily temperature of 33.3°C and the minimum of 6.1°C were recorded in March and in July, respectively. Although no stubble sample was assessed in July, the weather and soil records for this month were recorded in this research.

In the first 5 months of burial, the stubble retrieved from the two soil types showed no significant difference in percent weight reduction; however, from August the weight of the stubble buried in the field soil decreased more than that in the sand (Table 6.1). After 13 months of burial, the

stubble weight had decreased by 53.7% and 22% in the field soil and in sand, respectively. The percent decrease of stubble weight peaked (53.7%) after 13 months of burial (February 2005) in the field soil; however, the greatest reduction of stubble weight in the sand (28.6%) was observed after 7 months (August). From February (summer) 2004 to February 2005, a significant decrease in stubble weight was often observed every 2 months in the field soil. In sand, significant decreases in stubble weight occurred from March to May, in August and in January.

Figure 6.2. The daily average temperature in the field soil and sand from March 2004 to February 2005, recorded by Hastings Data Loggers.



Stubble weight decreased more rapidly in field soil than sand and the rate of decrease of stubble weight peaked at 4.9 g per month in August in the field soil and 4.1 g per month in May and August in sand (Figure 6.3). The rate of decrease of stubble weight was greater in field soil than in sand throughout the sampling period. The first sign of decomposition of the stubble, disappearance of the epidermal layer of the canola stem, was observed for samples from both soil

types in April. In November and thereafter, the stem pith was totally decayed in the field soil and some broken stubble was observed in the bags, whereas the pith of the stubble was only partially decomposed in sand.

Table 6.1. Percent decrease in weight of canola stubble buried in field soil or sand over 13 months relative to fresh weight prior to burial.

	Sampling time (month)[1]											
Soil type	Feb	Mar	Apr	May	Jun	Aug	Sep	Oct	Nov	Dec	Jan	Feb
Field soil	1.3[2]	5.3	11.4	17.8	20	34.5	35.8	36.9	47.1	49.9	46.9	53.7
Sand	0.4	4.4	9.3	16.4	16.1	28.6	17.5	21.7	16.5	17.4	24.5	22

[1] No stubble sample was assessed in July.

[2] The means of percentage decrease of stubble weight in four replicate bags; LSD = 4.6; $P < 0.05$.

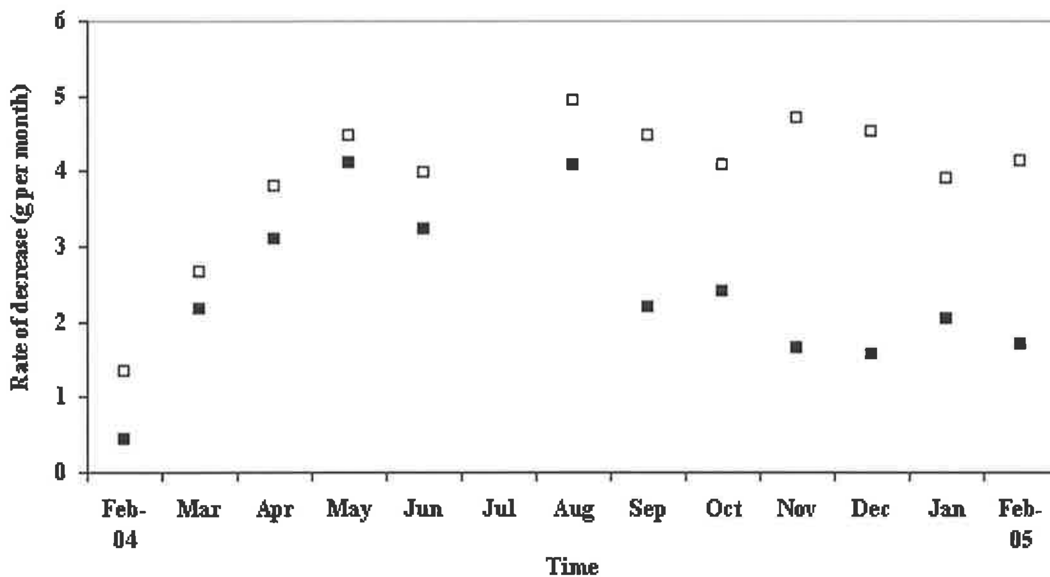


Figure 6.3. The rate of percent decrease of weight of stubble of canola in field soil (□) and sand (■) over 13 months; no stubble sample was assessed in July.

6.3.2 *L. maculans* and associated mycobiota on buried canola stubble

In January 2004, prior to burying the stubble in soil, 20 genera of fungi were isolated from 30 pieces of the stubble (Table 6.2). *L. maculans* was isolated from 70% of the stubble pieces, *Alternaria* spp. from 81.7%, *Stemphylium botryosum* (DAR 77420) from 60% and *Stachybotrys chartarum* from 26.7%. All fungi isolated from stubble before burial were also isolated during the sampling period. In total, 36 genera of fungi were isolated from the stubble that had been buried in the field soil and in sand (Table 6.2). *L. maculans*, which was isolated most frequently after *Alternaria* spp. in January 2004, showed a decreasing linear trend with time, except for an increase in October that resulted in a quadratic response. The frequency of isolation of *L. maculans* and *Alternaria* spp. decreased by 63.3 (from 70% to 6.7%) and 77.1%, respectively, from January 2004 to February 2005 and no difference was observed between the two soil types. As for *L. maculans*, mean isolation frequencies of *Alternaria* spp. decreased over time, except for an increase in September. *Alternaria alternata* (DAR 77414) was the species of this genus most frequently obtained from the stubble throughout the experiment. In January 2004, *Fusarium* spp. were isolated from 16.7% of the 30 pieces of the stubble. The mean isolation frequency of *Fusarium* spp. increased to 58.3% in March and fluctuated between 27.5 and 78.3% for the remainder of the study, regardless of soil type. *F. equiseti* (DAR 77416) was the predominant *Fusarium* species on the stubble and *F. acuminatum* (DAR 77415), *F. lateritium*, *F. sambucinum* (DAR 77417) and *F. xylarioides* were also isolated on DV8A medium.

Table 6.2. Percent isolation frequency of fungi from canola stubble buried in field soil and sand over 13 months.

Genus	Soil type[1]	Sampling time (month)[2]													L[3]	Q	LSD[4]
		Jan[5]	Feb	Mar	Apr	May	Jun	Aug	Sep	Oct	Nov	Dec	Jan	Feb			
Alternaria spp.	NS	81.7	82.9	45	31.7	25.4	25.4	22.5	35.8	10.8	11.3	9.6	4.6	4.6	S	S	12
Amerosporium sp.	NS	0	0	0	0	0.8	0	0	0	0	0	0.8	0	0.4	NS	NS	-
Arthrobotrys sp.	NS	0	0	0	1.2	0.4	33.7	7.1	12.9	1.7	25	15.4	7.5	9.2	S	S	9.6
Aspergillus spp.	NS	3.3	4.6	4.6	2.1	0.4	1.7	2.5	1.2	0.4	1.7	2.1	15.8	4.6	NS	NS	-
Bipolaris sp.	Field soil	0.8	0.8	10.8	2.5	0.8	4.2	10.8	2.5	0	1.7	0.8	1.7	2.5	NS	NS	3.8
	Sand		0	0	0	0	2.5	0	5.8	0	0	1.7	0	0			
Chaetomium sp.	NS	0	0	0.8	2.1	10	7.9	3.7	2.5	1.2	5.8	2.1	0	2.1	NS	S	4.4
Chloridium sp.	NS	0	0	0	0	0	0	0	0.8	0	0	0	0	0	NS	NS	-
Cladosporium sp.	NS	1.7	0.8	0	0	0	0	0	0	0	0	0.8	0	0.8	NS	NS	-
Coniothyrium sp.	NS	0	0	0	5	0	0	0	0	0	0	0.4	0	0	NS	NS	-
Coprinus sp.	Field soil	5	4.2	9.2	18.3	19.2	20	54.2	90	73	75.8	66.7	56.7	52.5	S	S	19.8
	Sand		3.3	2.5	3.3	5.8	0	0	0	10	15.8	11.7	6.7	7.5			
Curvularia sp.	Field soil	0	0	5.8	0	0	0	0	0	0	0	0	0	0	S	NS	0.9
	Sand		0	0	0	0	0	0	0	0	0	0	0	0			
Dendryphion sp.	NS	0	0	0	0	0	0	0	0	1.7	0	0	0	0	NS	NS	-
Fusariella sp.	NS	0	0	0	0	0	0.8	0	0	0	0	0	0	0	NS	NS	-

Fusarium spp.	NS	16.7	26.7	58.3	27.5	71.7	48.8	74.2	50.4	71.7	78.3	75	61.7	68.3	S	NS	17.5
Gliocladium roseum	Field soil	3.3	1.7	5.8	4.2	7.5	3.3	8.3	8.3	3.3	5	2.5	1.7	5	NS	S	15.6
	Sand		6.7	19.2	38.3	35	25	36.7	36.7	20	28.3	15.8	19.2	15.8			
Heteroconium sp.	NS	0	0	0	0	0	0	0	0.8	1.2	0	0	0	0	NS	NS	-
Leptosphaeria maculans	NS	70	42.5	36.7	30	22.1	17.5	5.4	12.9	13.7	10.4	4.6	6.7	6.7	S	S	8.4
Macrophomina phaseolina	NS	5	2.5	1.7	7.5	0	6.2	4.2	1.2	4.6	1.7	2.9	6.7	4.6	NS	NS	-
Massarina sp.	NS	0	0	0	0	0	0	0	0.8	0	0	0.8	0	0	NS	NS	-
Monacrosporium sp.	NS	0	0	0	0	0	0	0	8.7	9.2	3.7	4.2	1.7	3.7	S	NS	5.4
Myrothecium sp.	NS	2.5	0	0	6.7	1.7	2.9	1.7	5	2.5	0.8	2.1	0.8	2.5	NS	NS	-
Papulospora spp.	Field soil	0	0	25	3.3	8.3	0	5.8	16.7	2.5	14.2	12.5	7.5	6.7	NS	NS	-
	Sand		11.7	0	4.2	2.5	14.2	0	2.5	0.8	1.7	5	0	1.7			
Penicillium spp.	NS	0.8	0	0.8	0.4	0	0.4	0.4	0.4	0.8	0	0.4	0.8	0.8	NS	NS	-
Periconia macrospinosae	Field soil	0	0	0	0	0	0	1.7	6.7	4.2	10.8	6.7	2.5	25	S	NS	6.6
	Sand		0	0	0	0	0	0	0	0	0	1.7	0	0			
Phomopsis sp.	NS	0	0	0	0	0	0	0	2.1	1.2	0	0	0	0.4	NS	NS	0.8
Pseudotorula sp.	NS	0	0	0	0	0	0	0	0.4	1.2	1.7	0	0	0	NS	NS	-
Rhizoctonia sp.	Field soil	10	21.7	8.3	18.3	19.2	54.2	79.2	20	55.8	11.7	10	11.7	11.7	NS	S	14.1
	Sand		15	5	5.8	5.8	10	76.7	17.5	25.8	15.8	14.2	7.5	8.3			
Rhizopus sp.	NS	10.8	17.1	19.2	20.8	18.3	36.3	38.3	44.6	54.2	34.2	30.8	24.6	24.6	NS	NS	-
Sclerotinia sp.	NS	5.8	2.5	1.7	7.5	0.8	0	0	0	0	0.8	0	0	0	NS	NS	-

Sordaria sp.	NS	2.5	5.4	2.9	2.5	0.4	1.7	0	2.5	0	1.7	2.5	0.8	2.9	NS	NS	-
Sphaeronaema sp.	Field soil	0	0	0	0	18.3	5.8	10.8	7.5	3.3	11.7	6.7	3.3	4.2	NS	S	6.5
	Sand		0	0	0	6.7	6.7	0.8	5.8	2.5	0	2.5	0	2.5			
Stachybotrys chartarum	Field soil	26.7	5.8	0	60.8	60.8	55	57.5	34.2	34.2	50	52	53.3	60	S	S	18.6
	Sand		26.7	0	88.3	83.3	68.3	98.3	98.3	94.2	80.8	69.2	94.2	90			
Stemphylium botryosum	Field soil	60	52.7	83.3	30.7	48	53.3	40.7	21.3	18.7	16	12.7	8.7	11.3	S	NS	25.9
	Sand		56.7	75.6	81.1	77.8	64.4	65.6	62.2	45.6	40	25.6	38.9	38.9			
Trichoderma spp.	NS	2.5	2.5	2.1	0.4	0	0.8	0	5.8	2.5	25.4	15	14.6	12.5	S	NS	11.3
Ulocladium spp.	NS	5.8	2.1	2.1	3.3	0.8	11.7	22.5	24.6	17.1	14.6	13.7	7.9	8.7	S	S	7.4
Unknown Coelomycete	Field soil	2.5	2.5	1.7	1.7	5	1.7	34.2	0	0	13.3	3.3	0	0.8	NS	S	4.7
	Sand		0	0	0	0	0	0	0	0	0	0	0	0			

[1]NS non-significant difference in isolation frequency of the fungus between sand and field soil.

[2]No stubble was sampled in July 2004.

[3]L refers to a linear response and Q a quadratic response of the mean isolation frequencies for each fungus; NS non-significant; S significant.

[4]Least significant difference of the mean isolation frequency for each soil type over time; if no difference between the soils, LSD of the mean isolation frequency over time; - indicates no significant difference between isolation frequencies of each fungus over time.

[5]Percent isolation frequencies of fungi from the preliminary assay in January 2004 before burying the stubble in soil, from 10 canola stubble pieces (three 1-cm pieces per stem) cultured on DV8 medium.

In January and February 2004, *Trichoderma* spp., mostly *T. aureoviridae* (DAR 77421), were isolated from 2.5% of the stubble pieces. The frequency of isolation of *Trichoderma* spp. peaked at 25.4% in November then decreased to the end of the study; soil type did not affect this trend (Table 6.2). *S. botryosum* survived on the stubble in sand although it was less common in December; however, in the field soil the fungus declined in a linear trend from April to the end of the experiment. In general, *S. botryosum* was isolated more frequently from stubble buried in sand than in soil. The mean isolation frequencies of *Coprinus* sp. did not change significantly over time in sand, whereas colonization of the stubble in the field soil increased by 85% from January to September and then decreased by 23.3% over the last 3 months of burial (Table 6.2). *Coprinus* sp. was isolated more frequently from stubble in the field soil than in sand. The frequency of isolation of *S. chartarum* from stubble in sand increased from January 2004 to February 2005, except for March (Table 6.2). In the field soil, *S. chartarum* increased over the same time in a quadratic trend due to the decreases in February-March and September-October. The mean isolation frequencies of *S. chartarum* were 13.3-64.1% greater for stubble in sand than in the field soil.

Periconia macrospinosa, which was not isolated from the stubble in the field soil until August, increased to 25% in February 2005 (Table 6.2). In contrast, this fungus was isolated from the stubble in sand only in November (1.7%). The mean isolation frequency of *Gliocladium roseum* changed little over time in the field soil; however, in sand the fungus increased by 33.4% from January to September and then fluctuated throughout the rest of the study. *G. roseum* was isolated more frequently from stubble in sand than the soil throughout the study. *Rhizoctonia* sp. was isolated from 10% of the 30 pieces of stubble in January 2004 (Table 6.2). In the field soil following burial, colonization of the stubble by this fungus increased to 79.2% in August and then fluctuated for the remaining sampling dates. The mean

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isolation frequency of *Rhizoctonia* sp. in sand showed two increases, to 76.7% in August and 25.8% in October. This fungus was often isolated more frequently from stubble in field soil than sand.

Colonization of the stubble by *Arthrobotrys* sp., *Chaetomium* sp. and *Monacrosporium* sp. was observed after burial and then fluctuated over the sampling period. Although these three genera were not isolated from the stubble before burial, the stubble recovered from field soil and sand showed no difference in the mean isolation frequencies over time. *Ulocladium* spp., mostly *U. atrum* (DAR 77422), peaked at 24.6% in September and decreased to 8.7% in February 2005, regardless of soil type. The isolation frequency of *Bipolaris* sp. from stubble in sand and in field soil differed over the sampling period. *Sphaeronaema* sp. colonized stubble more frequently in the field soil than in sand. An unknown Coelomycete (efforts to identify this species have been unsuccessful so far) was isolated from stubble only in the field soil and peaked at 34.2% in August. The other species, including *Macrophomina phaseolina* (DAR 77418) and *Phomopsis* sp. (DAR 77419), were occasionally isolated from stubble.

6.3.3 Effect of environmental factors

There was no clear relationship between the trends of isolation frequencies of fungi from the stubble buried in the two soil types and the daily average air temperature or daily rainfall (appendix 3). Likewise, the isolation frequency of fungi was not related to daily average soil temperature (appendix 3) for either soil type. Colonization of stubble by *L. maculans* from February to August decreased as the mean monthly temperature dropped from 25°C to 12.1°C and mean monthly rainfall ranged from 0.2 mm to 3.1 mm (Figure 6.4a). From September to February, isolation of the fungus tended to decrease more slowly when mean monthly temperature increased to 20.5°C. *Alternaria* spp. also decreased from February to May as

mean monthly temperature decreased to 13.7°C, and then showed a slow decrease over the next 9 months, except for September (Figure 6.4b). In contrast, recovery of *Coprinus* sp. significantly increased in the field soil from February to September as the mean monthly temperature decreased to 12.9°C and mean monthly rainfall increased to 2.7 mm, and then decreased over the next 5 months in summer (Figure 6.4c). Isolation of *Fusarium* spp., which fluctuated from February to October and then remained stable until February 2005, did not show any relation to monthly temperature and rainfall trends (Figure 6.4d). Fluctuations in recovery of *S. botryosum* from sand and field soil occurred regardless of changes in the mean monthly temperature or rainfall. However, recovery of the fungus from the field soil increased in March with increase of 0.7 mm in mean rainfall and decrease in mean monthly temperature from 25°C to 20°C (Fig. 6.4e). The recovery of ***S.*** *botryosum* (Figure 6.4e) and *S. chartarum* (Figure 6.4f) from sand and field soil fluctuated over the 13 months and there was no obvious relationship between isolation frequency and weather data over time.

6.3.4 Pseudothecium formation on buried canola stubble

6.3.4.1 Effect of storage

No significant difference was observed in the mean density of pseudothecia on stubble retrieved from sand between the two examination times, September 2004 (21 pseudothecia per 0.5 × 1 cm) and February 2005 (20.9, LSD = 6.7; P = 1). Likewise, storage at room temperature (approximately 22°C) from September 2004 to February 2005 did not affect the mean density of pseudothecia on stubble retrieved from field soil, 11.9 and 11.1 (LSD = 10.6; P = 0.9), respectively.

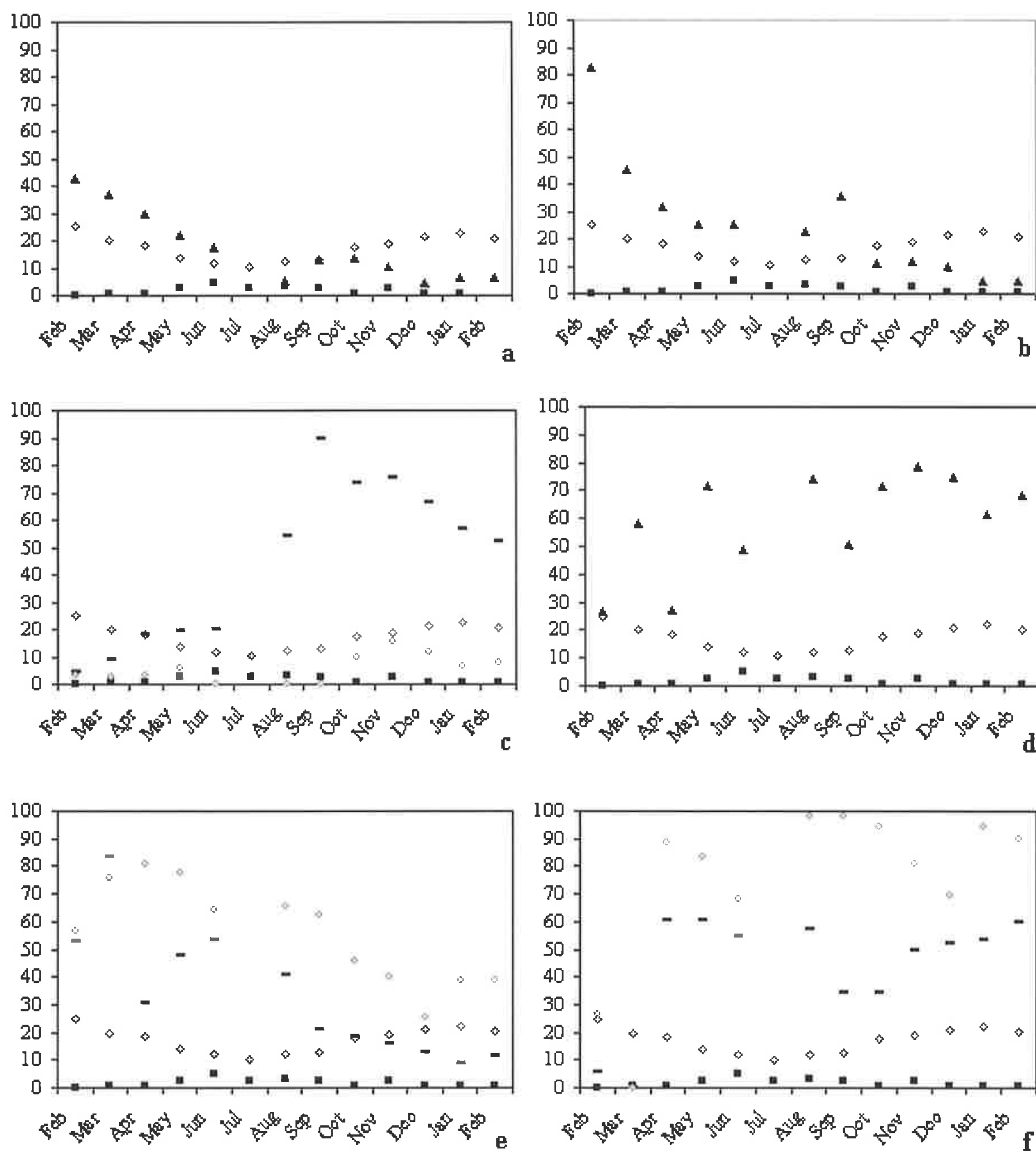


Figure 6.4. Percent isolation frequency of predominant fungi recovered from canola stubble buried in soil and mean monthly temperature, °C (◊) and rainfall, mm (■) over 13 months (no stubble was assessed in July); (▲) in sand and field soil when there was no difference, (-) in field soil, and (•) in sand; (a) *Leptosphaeria maculans*, (b) *Alternaria* spp., (c) *Coprinus* sp., (d) *Fusarium* spp., (e) *Stemphylium botryosum* and (f) *Stachybotrys chartarum*.

6.3.4.2 Effect of burial

At the time of sampling, pycnidia of *L. maculans* were observed on dark cankers on some of the stubble buried in sand from March to October and pseudothecia were evident on a few stubble pieces retrieved from May to October. Pycnidia were observed on stubble retrieved from field soil from May to September but no pseudothecia were observed over the 13 months.

The incubation time taken for pseudothecia of *L. maculans* on canola stubble to mature fluctuated between 25 and 30 days for stubble retrieved one month after burial, regardless of soil type (Table 6.3). The incubation time taken to reach stage A of maturation fluctuated between 10 days in February 2004 and 15 days in January 2005 for the stubble buried in sand, but to reach this stage took 15 days in February and 10 days in November in stubble retrieved from the field soil. The time taken to reach stage B fluctuated between 15 and 20 days over 12 months in sand, however, for stubble retrieved from the field soil this stage took the same time (20 days) in February and November. The time required for the other differential stages, C, D and E, fluctuated from February to the last sampling date for both soil types. The mean density of pseudothecia tended to decrease with increasing duration of burial in both soil types and pseudothecia failed to develop on stubble recovered from field soil and sand after November 2004 and January 2005, respectively. Pseudothecium density was greater on the stubble in sand than in the field soil in February 2004, March and August 2004 and no significant difference was observed at the other sampling times (Figure 6.5).

6.3.5 Pathogenicity of *L. maculans*

After moist incubation at 15°C, pseudothecia on stubble retrieved from both soil types produced ascospores that caused typical Phoma leaf spot lesions on canola plants over the

first 9 months of burial (Figure 6.6). There were insufficient pseudothecia or ascospores after this period to examine the pathogenicity of ascospores on canola seedlings.

Table 6.3. The incubation time (days) taken to reach differential stages (A, B, C, D, E) of pseudothecium maturation of *L. maculans* on blackleg-affected canola stubble buried in sand and field soil over 13 months.

Class		Sampling time (month)[1]										
		Feb -04	Mar	Apr	May	Jun	Aug	Sep	Oct	Nov	Dec	Jan -05
A	F[2]	15[3]	10	10	11.7	12.5	12.5	15	15	10	-[4]	-
	S	10	10	10	15	12.5	11.7	15	15	10	10	15
B	F	20	18.3	15	16.7	17.5	15	20	17	20	-	-
	S	15	16.7	16.7	15	20	16.7	20	17	15	20	20
C	F	21.7	23.3	20	20	25	22.5	22	22	25	-	-
	S	20	20	20	25	25	20	25	22	25	25	25
D	F	25	26.7	25	25	30	30	25	25	30	-	-
	S	25	25	26.7	35	30	25	30	25	30	30	30
E	F	30	30	25	26.7	35	35	32	30	35	-	-
	S	30	25	30	36.7	35	30	35	30	35	35	35

[1] No stubble was sampled in July; no pseudothecia developed on stubble in February 2005.

[2] F refers to field soil and S to sand.

[3] LSD = 2.6; $P < 0.05$.

[4] No pseudothecia developed on the stubble.

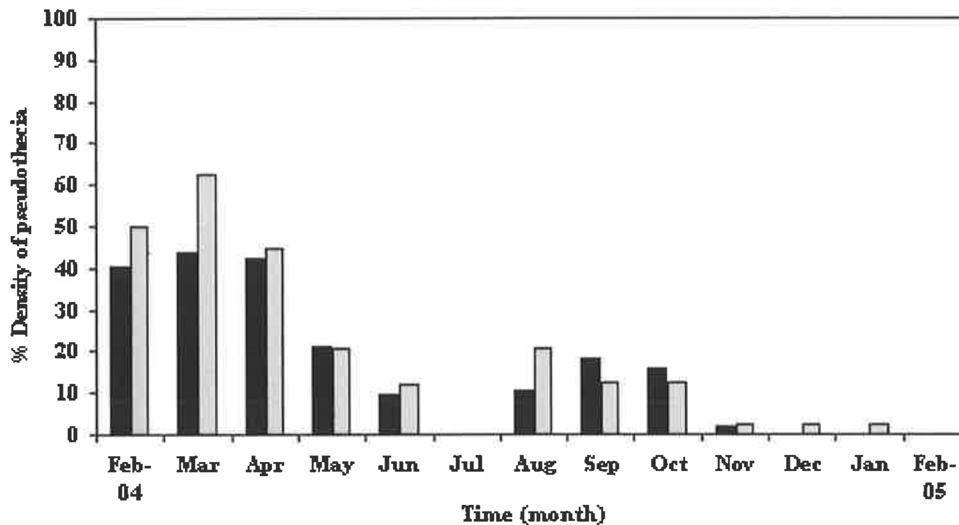


Figure 6.5. Density of pseudothecia (mean number of pseudothecia on 0.5 × 1 cm^2) of *L. maculans* on blackleg-affected canola stubble buried in sand (grey) and field soil (black) over 13 months and then incubated in moist conditions; LSD = 7.4; P < 0.05; no stubble was sampled in July.

6.3.6 Soil microbiota

In total, 16 genera of fungi were isolated from the field soil on PRA medium from February 2004 to February 2005 (Table 6.4). The total mean CFU (accumulated means of CFU of 12 sampling times for each fungus) of *Fusarium* spp., 316 × 10^2, *Penicillium* spp., 116 × 10^2, *Stachybotrys* sp., 112 × 10^2, and *Coprinus* sp., 110 × 10^2 per gram soil were generally larger than other genera isolated over the 13-month period. *Stemphylium botryosum* (6 × 10^2), *Ulocladium* sp. (8 × 10^2) and *Periconia* sp. (8 × 10^2) showed the lowest total mean CFU per gram soil over the 13 months. In addition, total number of CFU of all genera per gram soil in each month decreased from February to June 2004 and from August 2004 to January 2005. Despite the increase in August, the total population of fungi decreased by 148 × 10^2 CFU per gram soil over the 13 months.

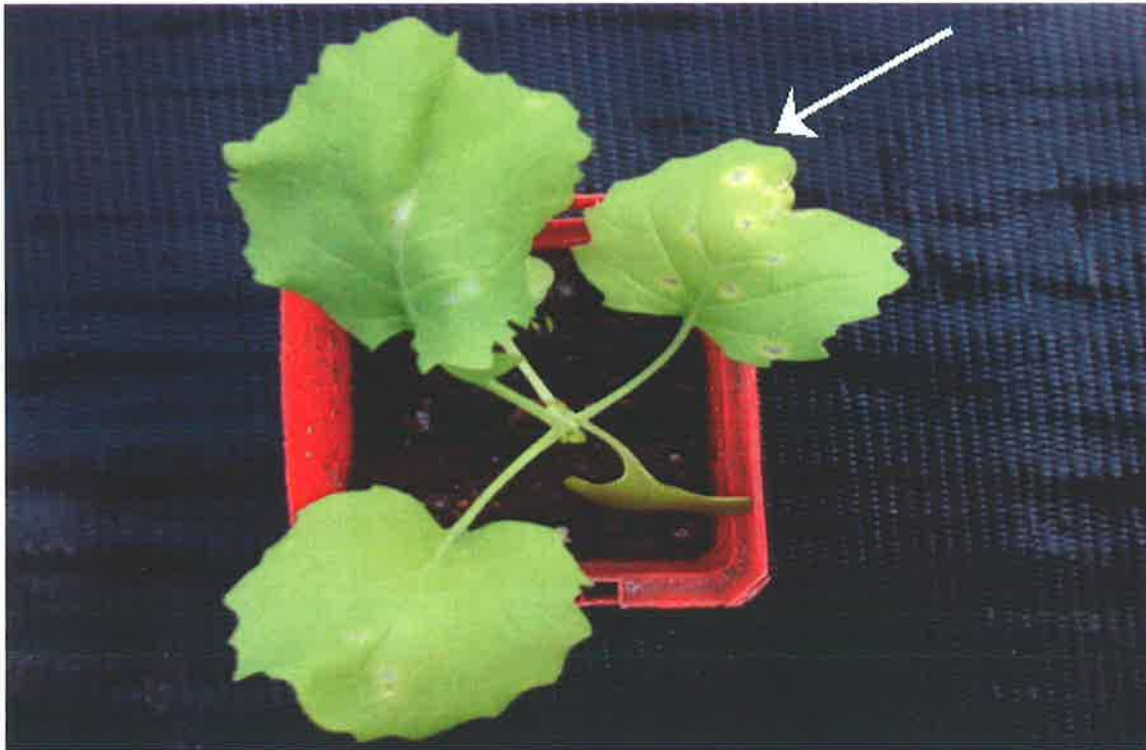


Figure 6.6. Phoma leaf spot (arrow) on canola cv. Karoo 15 days after inoculation with *L. maculans* ascospores from pseudothecia on stubble buried in soil.

Fusarium spp. decreased ($P < 0.05$) from February to November and then increased at the last sampling date, so that the population of this genus was greater in February-April and in February 2005, with 0.2-0.9 mm mean monthly rainfall, than the other sampling times. The CFU of *Coprinus* sp. and *Phoma* spp. per gram soil decreased ($P < 0.05$) from February 2004 to February 2005, although fluctuated somewhat over time. The population of *Stachybotrys* sp. peaked in April, decreased then showed little change over the remainder of the study. *Cladosporium* sp. was isolated only at the first and last sampling times. *Trichoderma* spp. and *Gliocladium* sp. were first isolated in the second month and their populations did not change in March-April then fluctuated over time. *Alternaria* spp., *Aspergillus* spp., *Penicillium* spp. and *Rhizoctonia* spp. were often isolated from field soil, but their population changed little

over the sampling dates. Other fungi were occasionally isolated from the field soil and their populations changed little throughout the experiment (Table 6.4).

Table 6.4. Average fungal populations (CFU × 10^2 per gram soil) in the field soil isolated on PRA medium over 13 months.

Genus	Sampling time (month)[1]											
	Feb -04	Mar	Apr	May	Jun	Aug	Sep	Oct	Nov	Dec	Jan	Feb-05
Alternaria spp.	6[2]	4	2	6	0	6	0	0	2	0	4	6
Aspergillus spp.	4	12	4	8	0	12	0	4	2	2	10	6
Cladosporium sp.	30	0	0	0	0	0	0	0	0	0	0	2
Coprinus sp.	32	14	14	4	2	16	4	2	4	0	12	6
Fusarium spp.	90	54	50	22	4	10	8	8	8	18	16	28
Gliocladium sp.	0	22	22	10	0	12	8	0	12	0	0	4
Myrothecium spp.	14	2	6	0	0	0	0	2	2	0	0	2
Penicillium spp.	20	14	8	10	2	12	6	10	8	8	10	8
Periconia sp.	0	0	4	0	0	2	0	0	0	0	0	2
Pestalotia sp.	0	8	0	0	0	6	0	0	0	0	0	2
Phoma spp.	26	4	4	4	2	4	2	2	0	0	2	0
Rhizoctonia spp.	0	10	6	8	4	4	0	0	2	2	6	2
Stachybotrys sp.	14	20	30	6	2	14	2	2	6	6	4	6
Stemphylium botryosum	0	2	0	0	0	0	0	0	0	0	0	4
Trichoderma spp.	0	22	22	6	0	14	0	0	0	0	6	6
Ulocladium sp.	0	0	0	0	0	2	0	0	0	0	2	4
Total	236	188	172	84	16	114	30	30	46	36	72	88

[1] No soil sample was assessed in July.

[2] Least significant difference of the means of five replicate plates per month was 21.2; $P < 0.05$.

Table 6.5. Average bacterial populations (CFU × 10^4 per gram soil) in the field soil isolated on CVA medium over 13 months.

Colony type	Gram reaction	Sampling time (month)[1]											
		Feb -04	Mar	Apr	May	Jun	Aug	Sep	Oct	Nov	Dec	Jan	Feb -05
1	−	2[2]	18	6	0	8	34	0	0	12	4	0	0
2	+	6	4	2	6	8	18	12	0	24	0	4	0
3	+	2	28	2	8	4	6	10	2	18	6	8	8
4	+	6	34	0	10	8	8	2	2	0	6	4	6
5	+	0	0	0	0	0	2	18	6	10	0	8	0
6	+	0	14	0	20	0	8	16	0	20	0	0	0
7	−	10	4	2	6	6	2	200	0	0	6	0	4
8	+	0	2	0	14	0	0	4	0	16	0	0	0
9	−	0	8	0	12	0	0	190	0	0	0	0	4
10	−	2	0	6	0	2	0	0	0	10	0	0	0
11	+	0	0	0	8	0	0	0	2	14	0	6	0
12	−	0	0	0	0	0	0	0	0	0	0	0	2
Total		28	112	18	84	36	78	452	12	124	22	30	24

[1] No soil sample was assessed in July.

[2] Least significant difference of the means of five replicate plates per month was 29.

In total, 12 morphologically different colony types of bacteria, seven Gram-positive (G+) and five Gram-negative (G−), were isolated from the field soil on CVA (Table 6.5). The population of bacteria isolated on CVA peaked at 452 × 10^4 CFU per gram soil in September 2004, when mean monthly rainfall of 2.7 mm and mild temperatures (8-17.5°C) were recorded. The other major increases occurred in March, May, August and November, when mean monthly rainfall increased by 0.6-2.5 mm. The mean CFU of two G− bacteria, colony types no. 7 and no. 9, were significantly larger ($P < 0.05$) than those of other bacteria in

September. These two bacteria also showed the largest total CFU (accumulated means of CFU of 12 sampling times for each colony type of bacteria). Two G– bacteria, colony types no. 12 and no. 10, showed the smallest total population over time. The population of another G– bacterium, colony type no. 1, increased to 34×10^4 CFU per gram soil in August, with mean monthly rainfall of 3.1 mm and mild temperatures (7.9-16.2°C), and decreased towards the end of the experiment. The approximate CFU of one G+ bacterium, colony type no. 4, decreased by 30×10^4 per gram soil from March 2004 to January 2005. The other bacteria showed little change over the sampling period. Total populations of G+ and G– bacteria isolated on CVA from February 2004 to February 2005 were 600 and 636×10^4 CFU per gram soil, respectively.

The population of fungi cultured from the field soil on SEA medium decreased from February to June, then increased in August-September and in November (Figure 6.7). The population of bacteria and total population of fungi and bacteria obtained on SEA decreased from February to June, and increased in August-September, November and January-February, with maxima of 680×10^4 and 1162×10^4 CFU per gram soil, respectively, in September. The population of bacteria was generally larger than that of fungi.

Total fungal populations from the field soil isolated on PRA and SEA fluctuated in a similar pattern over the sampling period, except in May and October (Table 6.6). However, the populations of fungi isolated on SEA were markedly greater than the total fungal population isolated on PRA throughout the experiment, e.g. the population of fungi on SEA was 202 times greater than that on PRA in February 2004.

Figure 6.7. Average fungal and bacterial populations (CFU × 10^4 per gram soil) in field soil isolated on SEA medium over 13 months.

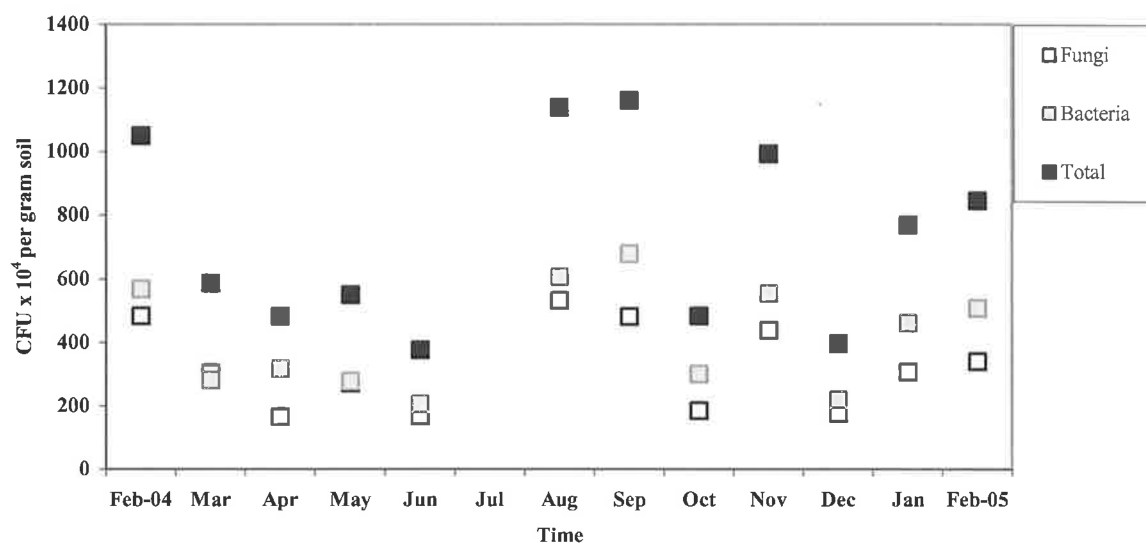


Table 6.6. Total fungal populations (CFU × 10^4 per gram soil) in the field soil on PRA and SEA over 13 months.

Medium	Sampling time (month)[1]											
	Feb-04	Mar	Apr	May	Jun	Aug	Sep	Oct	Nov	Dec	Jan	Feb-05
PRA	2.4	1.9	1.7	0.8	0.2	1.1	0.3	0.3	0.5	0.4	0.7	0.9
SEA	485	305	168	273	170	533	482	185	440	178	308	340

[1] No soil sample was assessed in July.

Total bacterial populations from the field soil isolated on CVA and SEA showed similar trends over the sampling period excepting February-May and in February 2005 (Table 6.7). For example, the total population of bacteria isolated on CVA increased from February 2004 to March, whereas the bacterial population on SEA decreased at the same time. Markedly

larger populations of bacteria were isolated on SEA than those on CVA throughout the experiment.

Table 6.7. Total bacterial populations (CFU × 10^4 per gram soil) in the field soil on CVA and SEA over 13 months.

Medium	Sampling time (month)[1]											
	Feb-04	Mar	Apr	May	Jun	Aug	Sep	Oct	Nov	Dec	Jan	Feb-05
CVA	28	112	18	84	36	78	452	12	124	22	30	24
SEA	568	282	318	278	208	608	680	300	555	220	462	508

[1] No soil sample was assessed in July.

6.4 Discussion

In this study, the isolation frequency of *L. maculans* from canola stubble decreased by 63.3% after 13 months of burial, whereas in a similar study by Baird *et al.* (1999) in the USA, the isolation frequency of the fungus decreased by 82% in just 5 months. Differences in environmental conditions, saprophytic microbiota or in the population structure of the pathogen in the two regions may have contributed to this difference in survival. Burial of blackleg-affected stubble increased the incubation time required for pseudothecia of *L. maculans* to mature and pseudothecium formation ceased earlier on stubble retrieved from the field soil than sand. Therefore, inoculum production of the pathogen on stubble, which is the main source of nutrient and substratum for the sexual stage of *L. maculans*, appeared to be influenced by soil factors. Five months of storage at room temperature did not appear to affect pseudothecium formation by *L. maculans* on stubble. Although *L. maculans* was isolated from canola stubble 13 months after burial, the fungus was unable to produce pseudothecia

and ascospores. This finding indicates the importance of burying stubble in soil in order to minimize production of ascospores in the field.

In Canada, Karbanda & Ostashewski (1997) observed pseudothecia on the stubble of highly susceptible cultivars upon retrieval from field soil one month after burial and on stubble of moderately resistant cultivars 10 months after burial. However, in the present research, no pseudothecia were observed on the stubble (before moist incubation) of a mixture of cultivars of canola retrieved from field soil in pots after 10 months and only pycnidia were observed on stubble sampled after 4 months. Pseudothecia developed on the stubble from field soil only after moist incubation in the laboratory, whereas pseudothecia were observed at the time of sampling after 4 months of burial in sand. Huang *et al.* (2003a) reported pseudothecium production on 40% of canola stem bases that had been buried in sand for 12 months then exposed to ambient conditions for 2-4 months in summer-autumn in the UK. In this research, pseudothecium formation (after moist incubation) ceased after 12 months of burial in sand and 10 months in the field soil. In addition, after 9 months, the pathogenicity of ascospores could no longer be examined due to insufficient pseudothecia and ascospores that developed on the stubble from both soil types. Therefore, ascospore production occurred over a shorter period on the blackleg-affected stubble in the present study. Comparing these findings with the results of British and Canadian studies suggests that differences in environmental conditions, such as climatic or soil factors and microbiota in soil or on stubble, may have resulted in differences in the ability of *L. maculans* to produce inoculum, and this merits further research.

Baird *et al.* (1999) observed a rapid decline of *L. maculans*, *Alternaria* spp. and *Fusarium* spp. in the course of their study and suggested a possible role for *Trichoderma* spp. in reduced survival of these fungi on the stubble buried in soil. Although the frequency of isolation of *L.*

maculans and *Alternaria* spp. from canola stubble decreased over time in the present study, the frequency of isolation of *Fusarium* spp. increased substantially over the sampling period. In addition, *Fusarium* spp. showed the second greatest isolation frequency, after *S. chartarum* in sand, at the end of the study. The ability of *Fusarium* spp. to grow and establish rapidly, sporulate abundantly and degrade cellulose (Domsch *et al.*, 1980) may be partially responsible for decreasing the colonization of stubble by *L. maculans*, *Alternaria* spp., *S. botryosum* and *G. roseum* at the last sampling time. *S. chartarum* has a worldwide distribution, mainly on dead plant material, and is common on rotting plant remains due to its abundant sporulation, ability to grow at 2-40°C, utilize and degrade a wide range of organic materials including cellulose, pectin, chitin and wood, and its antagonistic activity against numerous fungi (Domsch *et al.*, 1980). *S. chartarum* has been reported to produce metabolites which inhibit a wide range of fungi such as *Alternaria alternata*, *Aspergillus* spp., *Chaetomium* sp., *Curvularia* sp., *Fusarium* spp., *Macrophomina phaseolina*, *Myrothecium* sp., *Penicillium* spp., *Rhizoctonia solani*, *Sclerotinia sclerotiorum* (Rai & Saxena, 1975), *Sordaria* sp. and *Trichoderma viride*, as well as some actinomycetes and bacteria such as *Bacillus subtilis* in culture media (Butt & Gaffar, 1972). In view of the antagonistic activity of *S. chartarum* to other fungi and its ability to degrade cellulose and lignin, increased colonization of stubble in sand (by 90%) and field soil (by 60%) in the last sampling date may have influenced the survival of *L. maculans* and the other fungi on the stubble.

In the field soil, *Coprinus* sp. fluctuated throughout the experiment, but was most common after *S. chartarum* and *Fusarium* spp. at the final sampling date. The ability of *Coprinus* spp. to colonize cellulose and lignin (Redhead & Traquair, 1981) and to antagonise some fungi (Ershova *et al.*, 2001) enable it to compete with other fungi on plant debris for nutrients and space. Therefore, increasing colonization of the stubble in the field soil by this fungus could

contribute to the greater reduction of stubble weight in field soil than sand. In addition, *Coprinus* sp. appeared to colonize stubble more rapidly than other fungi, as its frequency of isolation in field soil increased by 85% within 8 months. This is in contrast with findings of the short-term study of Blenis & Chow (2005) that showed no significant recovery of *Coprinus* spp. from non-sterilized canola stubble inoculated with other test fungi. In this study, the presence of *Coprinus* sp. did not appear to affect the recovery of *L. maculans* from the stubble in field soil, because isolation frequency of *L. maculans* from stubble in sand and soil did not differ. This is consistent with findings in Canada (Blenis & Chow, 2005) that fungi (isolated from wood or canola stubble), including *Coprinus* spp., when co-inoculated on nonsterile stubble with *L. maculans*, neither eliminated the pathogen, nor contributed to its recovery. Blenis & Chow (2005) suggested that these fungi failed to become established in the stubble. The decrease of *Coprinus* sp. from September to February on stubble in the field soil could be due to the decrease of soil moisture with increasing temperature and decreasing rainfall, which are not favourable to this fungus (Redhead & Traquair, 1981; Yadav, 1988), or to sensitivity to surface disinfesting as the stubble decayed.

The frequency of isolation of *Rhizoctonia* sp. from stubble in sand and field soil peaked in August, and this fungus was most common after *S. chartarum* at this time in sand, however, *Rhizoctonia* sp. had declined by the last sampling date. This decline may reflect antagonistic activity of *S. chartarum*, *G. roseum* and *Trichoderma* spp. (Ehteshamul-Haque & Gaffar, 1991).

P. macrospinosae, a soil resident, strongly decomposes chitin and xylan, which may have contributed to the increased isolation frequency at the end of the experiment. *Ulocladium* spp., which is able to decompose cellulose (Domsch *et al.*, 1980), increased from June to

December. However, the maximum frequency of isolation, 24.6%, indicates less competitive ability than the other cellulose decomposers associated with stubble.

Arthrobotrys sp., a mycoparasitic and nematode-capturing fungus common in soil, colonizes partially decomposed plant material and utilizes substrates such as cellulose, chitin and wood. *Arthrobotrys* sp. has a relatively high competitive saprophytic ability compared with other fungal nematode predators but is sensitive to antagonistic influences of various soil organisms. Growth of *Arthrobotrys* sp. is also affected by soil moisture content (Kouyeas, 1964), which could explain the increased colonization of stubble by this fungus in June, September and November when mean monthly rainfall was 2.7-4.8 mm. The increases of *Monacrosporium* sp. (September-October) and *Arthrobotrys* sp. (in June, September and November) on the stubble may reflect the presence of nematodes in the pots, since rain increased soil moisture. The presence of nematodes is related to rapid growth, formation of adhesive networks, and high competitive saprophytic ability of these two antagonists (Cook, 1963). Further studies could also include detection and quantification of nematodes.

Papavizas & Davey (1961) reported that nitrogen depletion decreased not only the competitive colonisation of buckwheat and oat straw pieces by *Rhizoctonia* spp., but also resulted in decline of mycelium in the colonized substrate. Enrichment of the substrate with nitrogen enhanced the saprophytic ability and survival of *Rhizoctonia* spp. *Ophiobolus graminis* also declined on wheat straw due to intensified microbial competition for available nutrients, mainly N (Papavizas & Davey, 1961). The colonization of canola stubble by various saprophytes and decomposing fungi discussed earlier may have resulted in nitrogen depletion in stubble. In addition, the increasing colonization of the stubble by *Coprinus* sp., *Fusarium* spp., *S. chartarum* and *Trichoderma* spp., indigenous soil fungi and saprophytes,

indicated low saprophytic ability of *L. maculans*. Therefore, it may be possible to control *L. maculans* by the manipulation of saprophytic microorganisms normally present on canola residue to allow saprophytes and potential antagonists to colonise and displace the pathogen from infested residue. This needs to be investigated in future studies on survival of this pathogen on canola stubble left in the field after harvest.

Stubble buried in sand underwent 2.4 times as much decay as stubble in field soil by the end of the study. Soil structure and soil texture influence the turnover of organic matter through their impact on microbial decomposition processes. The microbiota is not only the main biodegradation agent in soil, it is also an important pool through which most of the organic matter in soil passes (Van Veen & Kuikman, 1990). Greater decomposition of stubble in field soil than sand, could also clarify the role of soil microbiota in the survival of the pathogen, as sand (standard) had little or no microbiota initially. Previous research showed that the number of fungi was higher in the rhizosphere of melon, pepper, cucumber and watermelon with high EC than in soils with low EC. The ratio of bacteria to fungi in soils of all four crops was higher in soils with low EC than those with high EC (Kang *et al.*, 1993). In addition to the effect of soil EC on soil microbiota, this factor might influence the microorganisms on stubble buried in soil, as in this study, field soil EC (102.4 mg/L) was higher than EC of sand (44.8 mg/L), which may clarify the greater biodegradation of canola stubble in soil than in sand. Lack of isolation of the unknown Coelomycete from stubble in sand suggests that this fungus may have originated from field soil. Differences in frequency of isolation of *S. chartarum*, *S. botryosum*, *P. macrospinosae*, *Coprinus* sp., *G. roseum*, *Rhizoctonia* sp., *Sphaeronaema* sp. and *Bipolaris* sp. from stubble in sand and in field soil in February 2005 may indicate a role of soil factors (including microbiota, EC and texture) in colonization of stubble by these fungi that may, in turn, have influenced survival of *L. maculans*.

The significant decrease in population of soil fungi isolated on PRA over a year, particularly for *Fusarium* spp., *Coprinus* sp., *Phoma* spp. and *Stachybotrys* sp., may be due to nutrient depletion in the soil or other inappropriate conditions inside the pot. However, the total population of fungi isolated on SEA fluctuated over time and did not decrease by February 2005. In addition, the fungal populations isolated on PRA and SEA differed, which may reflect differences in composition of the two media, in that SEA may be more conducive to the growth of soil fungi or allow recovery of a larger range of fungi. There is always the possibility of exclusion of a fungal group or species by using a single cultural medium. Therefore, in the present study one medium more selective for fungi (PRA) or bacteria (CVA) and one more general-purpose medium (SEA), were used in order to recover a range of microbiota. The prominent difference between populations of bacteria isolated on CVA and SEA also indicates the importance of the composition of the culture medium used in soil microbiota studies. Crystal violet is used in CVA medium as a selective agent to suppress the growth of G+ and non-enteric bacteria, as G+ bacteria are more sensitive to this agent than G– bacteria (Asghari *et al.*, 1992; Denyer *et al.*, 2000). According to the results, seven G+ colony types with total population similar to that of G– ones, on CVA plates, may indicate the presence of a larger population of G+ bacteria in the field soil than those isolated on CVA. It was beyond the scope of this study to examine the unculturable microbial populations of soil and stubble.

The effect of environmental factors, such as soil temperature and moisture, on soil microbiota has been reported in the past. The decrease of total population of fungi isolated on PRA from

June to December, excepting August, with average monthly rainfall 0.4-4.8 mm, is consistent with the findings of Menon and Williams (1957), who reported greater total numbers of fungi at low than at high soil moisture content. In addition, on PRA the largest total fungal population was obtained in February, with the lowest average monthly rainfall (0.2 mm) throughout the experiment. Because the decrease and increase of total fungi occurred at a wide range of temperature, this soil factor does not appear to be related to the fluctuations over the sampling period. Another form of alteration of soil structure that affects decomposition of organic matter occurs following rewetting of a dried soil, in that remoistening the soil results in an increased availability of organic matter to decomposing organisms (Van Veen & Kuikman, 1990). Here, the total population of bacteria isolated on CVA generally increased in those months when mean monthly rainfall increased compared with the previous month. Therefore, the increases in bacterial population in the field soil could be the consequence of remoistening of the soil in pots following rainfall. In addition, a similar pattern of increases in total populations of fungi and bacteria isolated on SEA compared with CVA suggests that soil moisture regime influenced soil microbiota.

See Addendum 5.

Buried canola stubble was colonized by a variety of fungi known to be capable of activities such as degradation of plant material, antagonism and competition with other fungi. The role of soil microorganisms should also be considered, as some fungi isolated from stubble differed in isolation frequency between the soil and sand. Although no clear relationship between air temperature, rainfall, soil temperature and survival of the pathogen on stubble was obtained in the present study, further investigation of environmental factors is required. The role of stubble-associated fungi, individually and in combination, in the decline of *L. maculans* and its ability to produce inoculum warrants further study. In addition, further

studies could clarify the role of stubble-associated fungi in decomposition of blackleg-affected debris, the main source of primary inoculum in Australia.

Chapter 7. Interactions between *L. maculans* and associated fungi on canola stubble *in vitro*

7.1 Introduction

Studies of the mycobiota of buried canola stubble and associated soil yielded a number of fungi with the potential to accelerate decomposition of the stubble and/or to antagonise *L. maculans* (chapter 6). Microbial activities such as antagonism, stimulation of growth, competition and decomposition of the substrate may affect the sporulation and survival of *L. maculans*. A better understanding of the interactions between *L. maculans* and potentially antagonistic fungi on canola stubble could improve strategies to reduce inoculum of the pathogen and contribute to the control of blackleg.

Previously, Baird *et al.* (1999) examined the isolation frequency of *L. maculans* and associated fungi from naturally blackleg-affected canola stubble in the US over a year of burial in soil, and looked at the effects of burial, depth of burial and location. The isolation frequency of *Trichoderma* spp. increased to 27% over the period of their study, however, the relationship between *L. maculans* and *Trichoderma* spp. or other stubble-associated fungi was not investigated. In Canada, Blenis & Chow (2005) reported that none of 39 fungal isolates from wood or 17 from canola stubble (including *Cyathus olla*, *Coprinus* spp. and six unidentified isolates) were effective in eliminating *L. maculans* from stubble nor in causing significant decomposition of non-sterile stubble when co-inoculated with *L. maculans*, either under laboratory conditions or in the field. It was suggested that these fungi failed to become established in the stubble (Blenis & Chow, 2005). Therefore, management of blackleg disease through stubble decomposition needs further research to identify decomposers and antagonists of *L. maculans* that would be able to colonise stubble effectively.

Likewise, a better understanding of biocontrol agents active against *L. maculans* may help in developing more effective management strategies for blackleg. Researchers who have studied microbial antagonism of the pathogen have reported mainly bacterial antagonists. Kharbanda *et al.* (1999) reported that *Paenibacillus polymyxa* strain PKB1 significantly reduced germination and germ tube length of *L. maculans* pycnidiospores *in vitro* and suppressed blackleg development on canola when leaves were co-inoculated with a suspension of PKB1 cells and pycnidiospores. In field experiments, PKB1 reduced development of pseudothecia and ascospores, and decreased survival of the fungus on stubble (Kharbanda *et al.*, 1999). Zhao (2001) obtained 58 bacterial isolates from compost and the rhizosphere of different crops, all of which inhibited *L. maculans* on culture media. Colonization of seedlings, from seeds that had been inoculated with fluorescent pseudomonads and *Serratia plymuthica*, inhibited the pathogen more effectively than application of *Bacillus* strains (Zhao, 2001). A rapid assay was introduced for identifying potential biocontrol agents against *L. maculans*, using wounded cotyledons of cv. Westar (Ramarathnam & Fernando, 2002). This method identified three of 112 bacterial cultures isolated from leaves, stems and roots of canola plants as inhibiting *L. maculans* on culture media and on canola cotyledons.

A few studies have examined the antagonistic activity of fungi against the pathogen in the laboratory. A strain of *Penicillium verrucosum* was recovered as an aerial contaminant inhibitory to growth of *L. maculans* and *Rhizoctonia solani* (Karbanda & Dahiya, 1990). Karbanda & Dahiya (1990) isolated a yellow crystalline metabolite of *P. verrucosum* from culture filtrate of the fungus on potato dextrose broth that was identified as closely related to citrinin and which was highly inhibitory to *L. maculans*, *R. solani* and *Sclerotinia sclerotiorum*. *Cyathus striatus* was another fungus that showed potential as a biocontrol agent against *L. maculans* (Maksymiak & Hall, 2000). It was suggested that antibiotic compounds

produced by *Cyathus* spp. might be detrimental to *L. maculans* but needed experimental confirmation. Maksymiak & Hall (2000) reported that *Cyathus striatus* possessed a greater ability for primary resource capture by producing more cellulase and lignase than the other species of this fungus tested. Furthermore, application of the biopreparation "Supresivit®" based on spores of *Trichoderma harzianum* at 0.5 g per kg of a mineral fertilizer decreased the severity of Phoma leaf spot on canola in the field (Hysek *et al.*, 2002), such that the infestation of leaf area decreased by 10%.

As there is no reliable and economic chemical control available for blackleg, biological control offers a means of management of the disease. In addition, detection of effective decomposers of canola stubble capable of reducing the amount of inoculum in the field may facilitate disease control. As the effect of stubble-associated mycobiota on the pathogen is little understood, this research examined interactions between *L. maculans* and fungi isolated from canola stubble and field soil towards a better understanding of fungal antagonism of *L. maculans* and decomposition of stubble.

7.2 Materials and Methods

To investigate the interactions between *L. maculans* and associated fungi on canola stubble, three experiments were conducted, involving dual culture on agar in Petri dishes, agar-coated slides and stubble in the laboratory. In total, 35 fungal species isolated from canola stubble or field soil (listed in Tables 6.2 and 6.4), including *L. maculans*, were used in this study (Table 7.1). Single spore-derived cultures and vegetative cultures of these fungi were subcultured on PDA (appendix 2) and incubated at room temperature (approximately 22°C) under fluorescent light (Phillips TDL 36W/840, Thailand) and black light (NEC T10 40W/FL40SBL, Japan) for

up to 2 weeks to produce spores or hyphae for use as inoculum in the experiments described below.

7.2.1 Dual culture on agar plates

The effect of 34 fungal species on hyphal growth of *L. maculans* on agar (Table 7.1) was examined. A 1 mm^2 plug from the margin of a 2-week-old culture of each species and a 1 mm^2 plug of *L. maculans* on PDA were placed on PDA in 9 cm diameter Petri dishes, 4 cm apart (dual culture). Three replicate Petri dishes were prepared for each test species, and three Petri dishes were inoculated with one plug of *L. maculans* only as the control. Petri dishes were then incubated at room temperature as described in section 7.2, for up to 3 weeks, after which they were examined for changes in colony appearance, hyphal growth and sporulation of *L. maculans*. The type of interaction, such as inhibition of hyphal growth resulting in a small colonies, deformation or overgrowth of colonies, or formation of inhibition zones between the colonies was recorded. Representative cultures were photographed.

7.2.2 Dual culture on agar-coated slides

Sterile glass slides were placed in Petri dishes lined with moistened filter paper. One ml of 2% water agar (appendix 2) was placed on each glass slide to form a uniform layer (*ca* 1.5 × 3 cm). One plug, 1 mm^2, of spore-bearing mycelium from a single spore-derived culture or vegetative culture of each test species was transferred to one end of an agar-coated slide. On the other end, a similar plug of *L. maculans* was placed, 3 cm apart from the test fungus. Three slides were prepared and inoculated for each of 26 potential antagonists (Table 7.1). Three slides were inoculated with two plugs of *L. maculans* only as controls. Petri dishes were then incubated at room temperature (see section 7.2) for up to 2 weeks. Agar-coated

slides were observed microscopically at 4-day intervals to check the interaction between hyphae of *L. maculans* and the test fungus. If there was any change in hyphal growth and sporulation of *L. maculans* in dual cultures in comparison with the control, hyphae were photographed.

7.2.3 Inoculation of blackleg-affected stubble with fungi

Sterile sand (Waite Campus, The University of Adelaide, SA), 5 g, was added to each of four replicate 9 cm diameter Petri dishes for each test fungus and sprayed with sterile distilled water. Naturally infected canola stubble used in this experiment was collected in January 2004 (see section 2.1.3). Pieces of stubble having blackleg cankers were cut into 3-4 cm segments and placed on the sand (three segments per Petri dish). Spore suspensions of 21 fungal species (Table 7.1) were obtained from cultures on PDA (see section 7.2) and adjusted to 10^3 spores ml^{-1}. Two ml of suspension was pipetted onto each stubble segment so that it was covered by a film of suspension. For *Papulospora* sp. and *Coprinus* sp., two plugs, 1 mm^2 each, of PDA culture of the fungus and 2 ml of sterile distilled water were added to each stubble segment. For controls, each stubble segment in four Petri dishes was inoculated with 2 ml of ascospore suspension of *L. maculans* (see section 4.2.2.1). Petri dishes were then sealed with Parafilm M® (Alcan Inc., American National Can., USA) and incubated at 15°C under a 12 h photoperiod (see section 2.2.2). Pseudothecium formation was checked after 6 weeks of incubation and the density of pseudothecia (the number of pseudothecia in a 0.5 × 1 cm area) was assessed for each stubble segment. The mean density of pseudothecia per segment was calculated as the mean of densities of three replicate segments in four replicate Petri dishes (in total, a mean of 12 densities of pseudothecia per test species).

7.2.3.1 Effect of inoculant fungi on germination of *L. maculans* ascospores

After assessment of pseudothecium density, six stubble segments were randomly selected for each species and for *L. maculans*-only controls, to examine the germination of ascospores of *L. maculans*. To avoid collecting spores of other fungi in the ascospore suspension, stubble segments were rinsed in distilled water and left on clean filter paper to dry. Then stubble segments for a given species were attached to the underside of a Petri dish lid with Vaseline petroleum jelly in order to produce ascospore suspension, as described in section 4.2.2.1. Three 50 μl aliquots of ascospore suspension from each Petri dish were placed on glass slides inside Petri dishes lined with moistened filter paper (to maintain high relative humidity). The Petri dishes were then incubated at 20°C for 24 h. The germination of ascospores from stubble inoculated with each test fungus was examined microscopically. Whether ascospores of *L. maculans* on glass slides, following exposure to each species, had germinated (+) or not (-), was recorded.

7.2.3.2 Effect of spores of potential antagonists on germination of *L. maculans* ascospores

The presence of spores of the 21 test fungi in the ascospore suspension of *L. maculans*, obtained from stubble inoculated with each of species, and the effect of these associated spores on ascospore germination were examined. Three aliquots (0.5 ml) of the ascospore suspension, obtained above for a given combination, were spread evenly over a thin layer of PDA (one aliquot per Petri dish). Petri dishes were incubated at room temperature (see section 7.2) for 24 h and then examined microscopically. The presence of spores of other fungal species in the ascospore suspension and inhibition of germination of *L. maculans* ascospores were observed microscopically.

7.2.4 Effect of fungi on decay of canola stubble

Sterile sand, 5 g, was added to each of ten replicate Petri dishes for each of *Coprinus* sp. and *S. chartarum*, and for the control. Sand was moistened with 2 ml sterile distilled water. Canker-free pieces of stubble, as above, were cut into 3-4 cm segments and weighed individually. Stubble segments were surface sterilized in 0.5% sodium hypochlorite solution for 3 minutes and rinsed in sterile distilled water, then placed on the sand, one segment per dish.

Spore suspension of *S. chartarum* obtained from 2-week-old colonies on PDA was adjusted to 10^3 spores ml^{-1}. Two ml of spore suspension were pipetted onto each of ten stubble segments so that they were covered by a film of suspension. For *Coprinus* sp., two plugs, 1 mm^2 each, of PDA culture of the fungus were transferred to each stubble segment. For controls and for stubble inoculated with *Coprinus* sp., 2 ml of sterile distilled water were pipetted on each stubble segment. Petri dishes were then sealed with Parafilm M® and incubated at 20°C under a 12 h photoperiod (see section 2.2.2) for 2 months.

After the incubation period, stubble segments were removed from Petri dishes and weighed (fresh weight) after removing sand particles using a cotton bud. Stubble segments were left on clean filter paper at room temperature (approximately 22°C) for approximately 6 h to dry and then weighed individually (air-dry weight). The difference between the original (before inoculation) and final (fresh and air-dry) weights of each stubble segment and the mean difference of the ten stubble segments were calculated to determine the amount of stubble decay caused by the two test fungi compared to the control stubble.

7.2.5 Statistical analysis

The data for the density of pseudothecia formed on stubble inoculated with the 21 test species were subjected to ANOVA using GenStat version 8.2 (Lawes Agricultural Trust, Rothamsted Experimental Station, 2005).

7.3 Results

7.3.1 Dual culture on agar plates

Interactions between the test fungi and *L. maculans* on PDA are summarized in Table 7.1. *L. maculans* (the control) formed colonies of approximately 7 cm diameter and produced abundant black pycnidia (Figure 7.1a). An inhibition zone between colonies of the test fungus and *L. maculans* formed in dual culture with species such as *Alternaria* spp., *Cladosporium* sp., *Fusarium* spp., *Myrothecium* sp. and *Stachybotrys chartarum* (Figure 7.1b-c). The colony of *L. maculans* remained small on PDA compared with the control colony. Restricted colonies of *L. maculans* were partially to completely covered with growth of *Arthrobotrys* sp. (Figure 7.1d), *Macrophomina phaseolina*, *Monacrosporium* sp., *Penicillium* sp., *Phomopsis* sp. and *Sphaeronaema* sp. Growth of *L. maculans* colonies was inhibited at an early stage and then covered by fast-growing colonies of *Aspergillus* sp., *Sordaria* sp. (Figure 7.1e), *Trichoderma aureoviride*, and the unknown Coelomycete on PDA. Growth of *F. lateritium* and *F. sambucinum* caused deformation of *L. maculans* colonies, such that no pycnidia formed and the colony changed from dark olive colour to pale green, in addition to complete or partial coverage (Figure 7.1f). Mycelium of F. lateritium appeared to be sparse in the zone closest to L. maculans. Growth of *Fusariella* sp., *Stemphylium botryosum* and *Ulocladium atrum* had no effect on the growth of *L. maculans* colonies. *L. maculans* overgrew none of the test species.

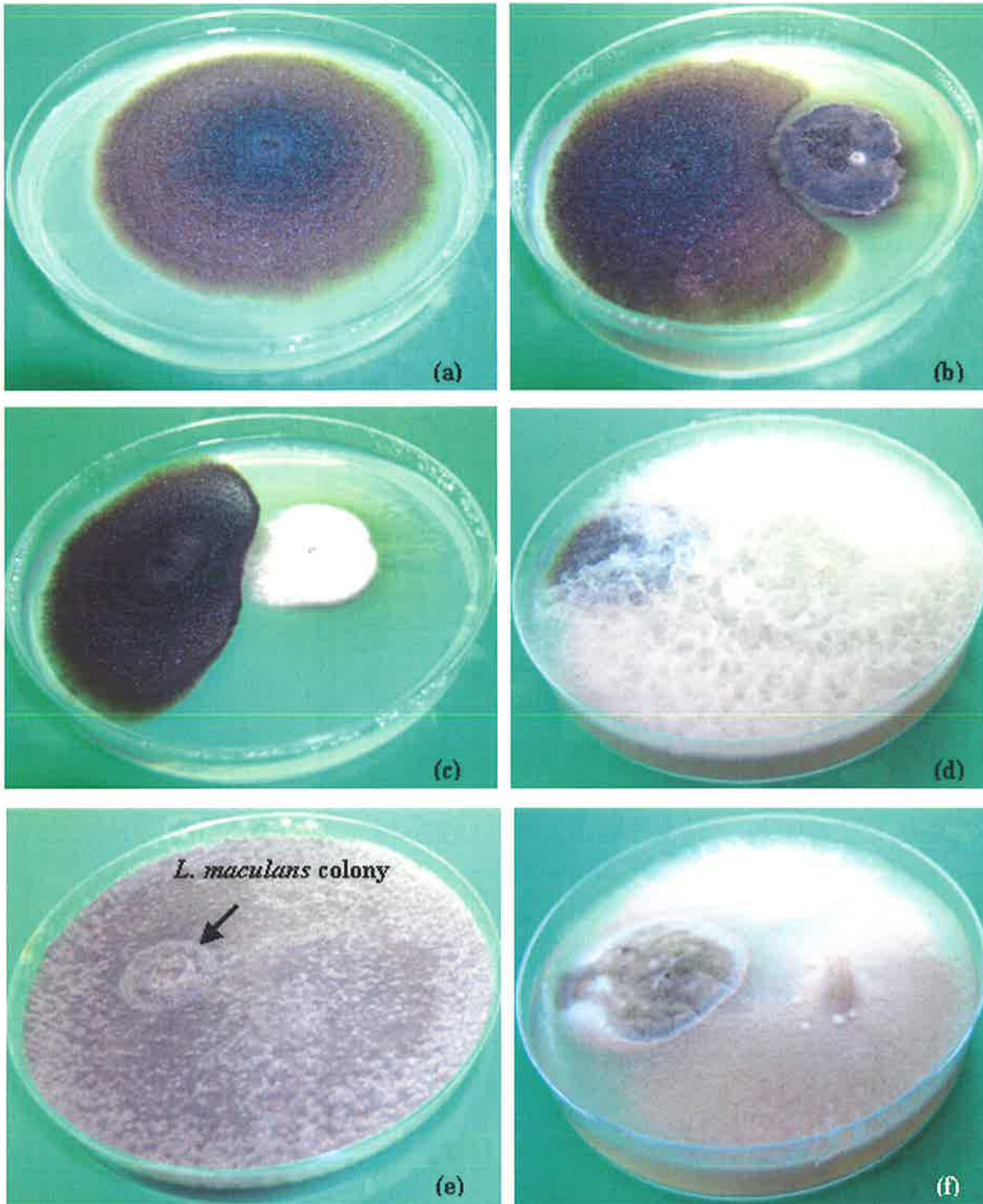
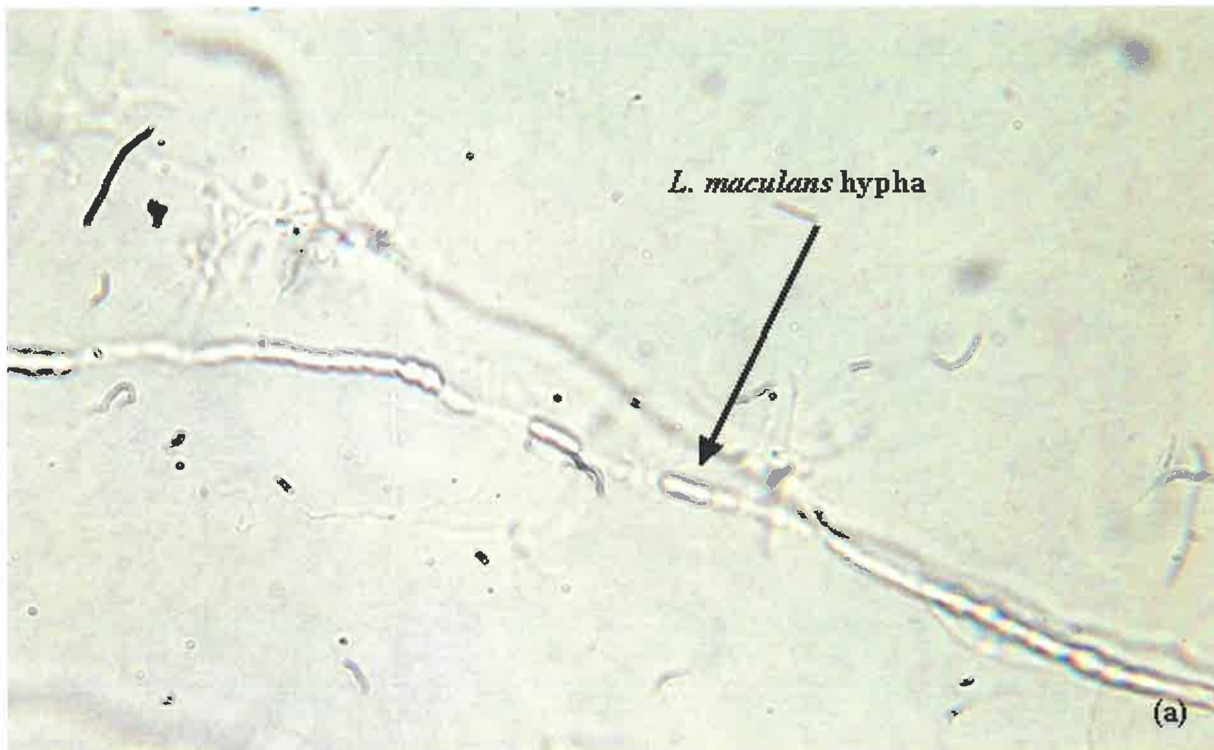
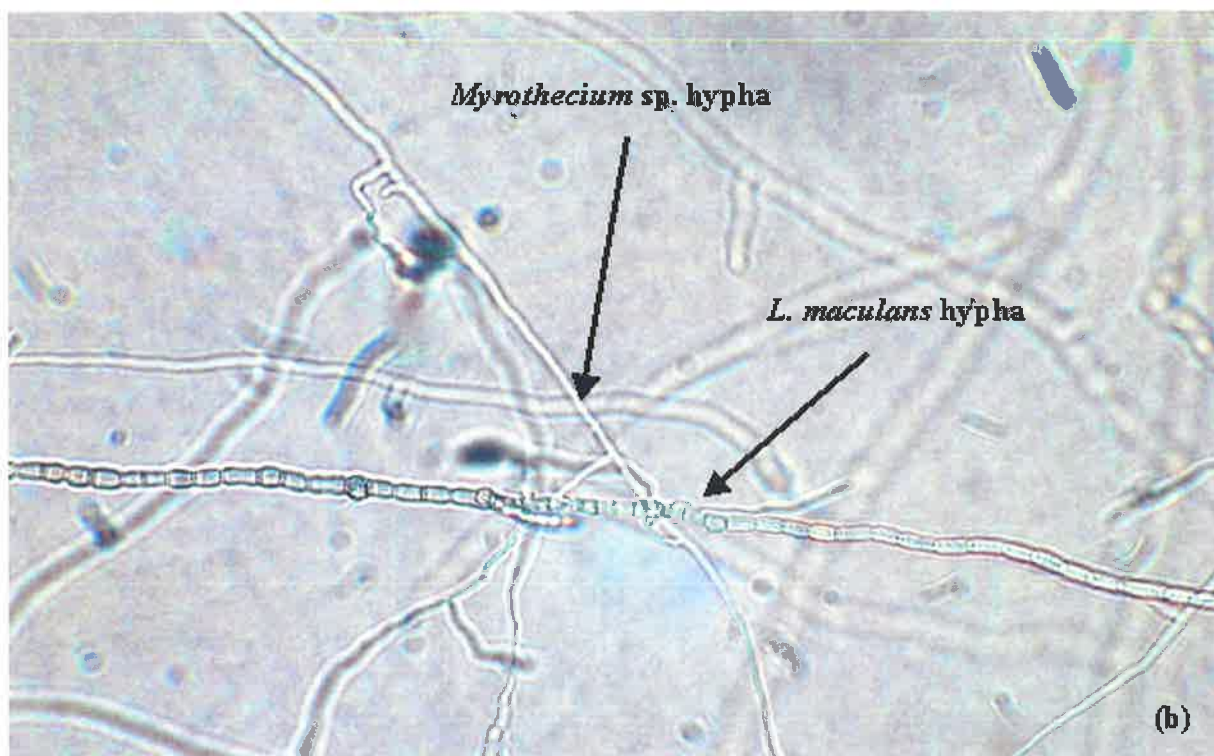
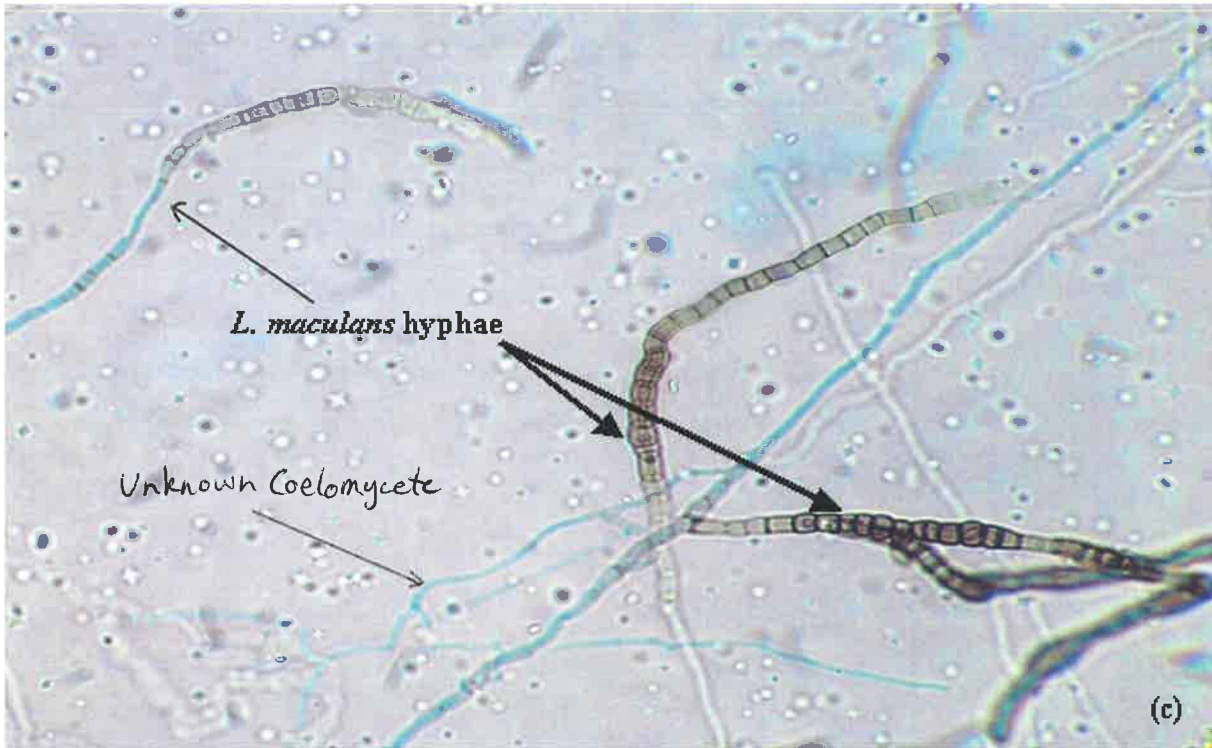


Figure 7.1. Types of interactions between *L. maculans* (on the left) and test fungi (on the right) in dual culture on PDA after 3 weeks at room temperature; (a) control, *Leptosphaeria maculans*, (b) inhibition zone in the presence of *Stachybotrys chartarum*, (c) inhibition zone in the presence of *Myrothecium* sp., (d) complete overgrowth by *Arthrobotrys* sp., (e) growth inhibition resulting in a small colony (arrow) in the presence of *Sordaria* sp., and (f) colony deformation in the presence of *Fusarium lateritium*.


L. maculans hypha
(a)


Myrothecium sp. hypha
L. maculans hypha
(b)



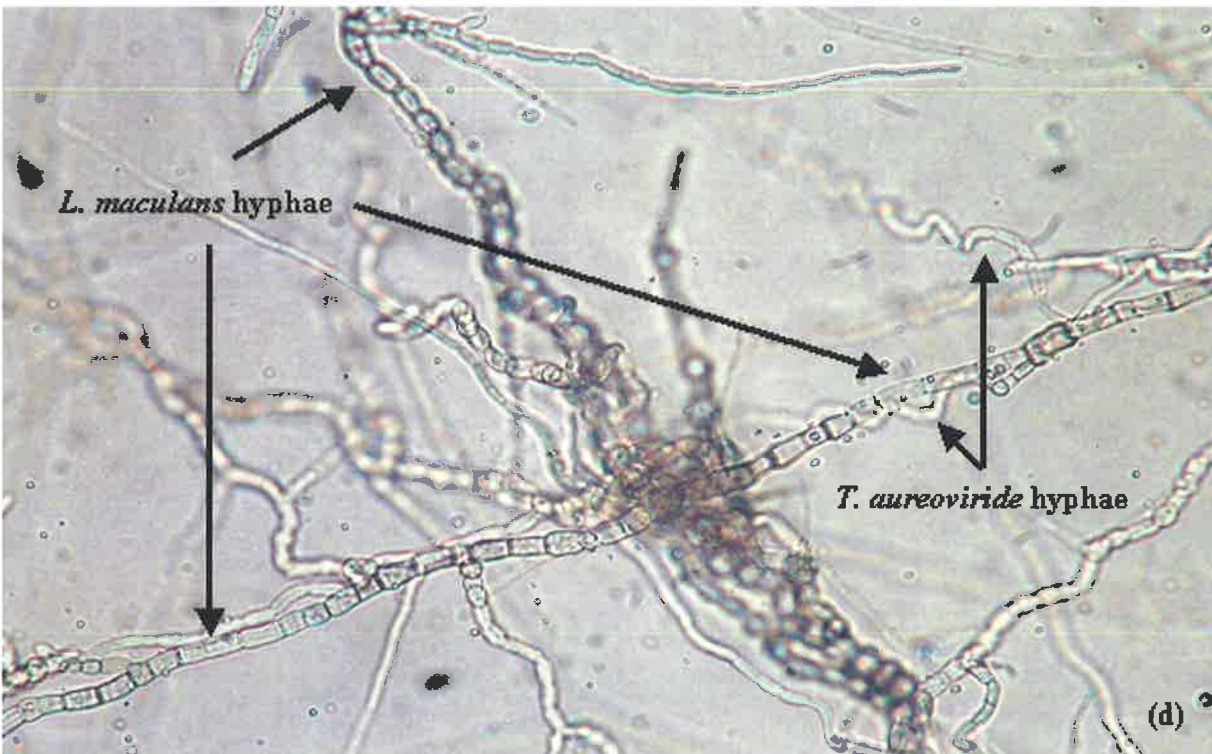


Figure 7.2. Types of interactions between hyphae of *Leptosphaeria maculans* and test fungi in dual cultures on water agar after 2 weeks at room temperature; lysis of *L. maculans* hyphae in the presence of (a) *Gliocladium roseum* and (b) *Myrothecium* sp.; deformation of *L. maculans* hyphae in the presence of (c) the unknown Coelomycete and (d) *Trichoderma aureoviride*.

7.3.2 Dual culture on agar-coated slides

Interactions between the 26 test fungi and *L. maculans* on water agar are summarized in Table 7.1. No sign of hyphal growth inhibition, inhibition zone, hyphal lysis and hyphal deformation was observed on controls of *L. maculans* alone. Inhibition of hyphal growth, resulting in small colonies of *L. maculans* on the slides, was only observed in dual cultures with *Arthrobotrys* sp. and *Aspergillus* sp., which grew faster than *L. maculans* in the water agar layer. An inhibition zone, an area in which no hyphae grew on the medium after 2 weeks, formed between colonies of *L. maculans* and ten test species, including *Alternaria* sp., *Bipolaris* sp., *Myrothecium* sp. and *Stachybotrys chartarum*. Lysis of *L. maculans* hyphae occurred when hyphae of *Gliocladium roseum*, *Myrothecium* sp. and *Penicillium* sp. grew over *L. maculans* hyphae. In the presence of hyphae of *Trichoderma aureoviride* and the unknown Coelomycete, hyphae of *L. maculans* were deformed, in that differentiation of hyphae to pycnidia was interrupted and hyphal cells remained swollen, closely septate and dark green colour. *L. maculans* was distinguished from the test species based on comparison of the morphology of hyphae (width, colour, branching pattern, sporulation) at the site of interaction with the potential antagonist (Figure 7.2).

7.3.3 Inoculation of blackleg-affected stubble with fungi

All control stubble and most stubble segments inoculated with the 21 test species produced mature pseudothecia after 4-5 weeks of incubation at 15°C. The mean density of pseudothecia in the control was 26.6. The density of pseudothecia was significantly reduced (LSD = 8.6; $P < 0.05$) in the presence of all inoculant fungi compared with the control, except *Alternaria alternata*, *Periconia macrospinosae*, *Sphaeronaema* sp. and *Stemphylium botryosum*. The mean pseudothecium density was least (3.6) in the presence of the unknown Coelomycete (Table 7.1). Stubble segments inoculated with spores of *F. equiseti*, *Gliocladium roseum*,

Sordaria sp. and *Trichoderma aureoviride* produced few pseudothecia. None of the species eliminated *L. maculans* from stubble.

Table 7.1. Interactions between *L. maculans* and selected fungi on PDA plates, on water agar-coated slides and on blackleg-affected stubble.

Genus or species	Colony morphology on PDA	Hyphal interactions on agar-coated slide	Pseudothecium density on stubble
Leptosphaeria maculans	Control	Control	26.6[1]
Alternaria alternata	IZ[2]	IZ	19.2
Alternaria sp.	IZ	IZ	13.7
Amerosporium sp.	PC	–	–
Arthrobotrys sp.	CC & GI	PC & GI	16.3
Aspergillus sp.	CC & GI	GI	14.3
Bipolaris sp.	GI	IZ	17.8
Chaetomium sp.	PC & GI	–	–
Chloridium sp.	IZ	–	–
Cladosporium sp.	IZ	–	–
Coprinus sp.	PC	PC	11.3
Curvularia sp.	IZ & GI	IZ	–
Fusariella sp.	0	–	–
Fusarium acuminatum	IZ	PC	–
F. equiseti	GI & PC	PC	8.8
F. lateritium	GI & PC	CC	–
F. sambucinum	GI & PC	PC	15.1
F. xylarioides	GI & PC	PC	–
Gliocladium roseum	PC	HL	7.8
Macrophomina phaseolina	CC & GI	–	15.2
Monacrosporium sp.	GI & PC	CC	–
Myrothecium sp.	IZ	IZ & HL	12.2
Papulospora sp.	PC	PC	10.7
Penicillium sp.	CC & GI	HL	13.3
Periconia macrospinosae	PC	0	21.5

Pestalochia sp.	PC	–	–
Phomopsis sp.	PC & GI	IZ	13.7
Pseudotorula sp.[3]	IZ	–	–
Sordaria sp.	CC & GI	CC	7.1
Sphaeronaema sp.	PC & GI	IZ	20.3
Stachybotrys chartarum	IZ	IZ	9.4
Stemphylium botryosum	0	IZ	22.5
Trichoderma aureoviride	CC & GI	HD	7.2
Ulocladium atrum	0	IZ	–
Unknown Coelomycete	CC & GI	PC & HD	3.6

[1] The mean density (the number of pseudothecia in 0.5 × 1 cm area) of *L. maculans* pseudothecia formed on 12 replicate stubble segments inoculated with each test fungus; LSD = 8.6; $P < 0.05$.

[2] CC = *L. maculans* colony was covered by test fungus on PDA amended with streptomycin; GI = inhibition of colony growth, HD = deformation, and HL = lysis of hyphae of *L. maculans*; IZ = formation of inhibition zone between the two fungi, PC = partial coverage of colony of *L. maculans*, 0 = test fungus had no effect, – = not tested.

[3] The colony of this species remained restricted on agar media and did not grow close to *L. maculans*.

7.3.3.1 Effect of inoculant fungi on germination of *L. maculans* ascospores

Germination of > 70% of ascospores was observed in ascospore suspensions from stubble, regardless of the inoculant fungus, after 24 h incubation at 20°C.

7.3.3.2 Effect of spores of potential antagonists on germination of *L. maculans* ascospores

Of the 21 species tested, only ascospores of *Stemphylium botryosum* were observed in the ascospore suspension of *L. maculans*. The ascospores of *S. botryosum* had no obvious effect on germination of *L. maculans* ascospores after 24 h of incubation on PDA.

7.2.4 Effect of fungi on decay of canola stubble

After incubation for 2 months, most stubble segments were partially covered with sporophores and spores of *S. chartarum*. Stubble segments inoculated with *Coprinus* sp. were partially covered with white mycelium. Average fresh and dry weight of ten stubble segments inoculated with *Coprinus* sp. and *S. chartarum* decreased more than that of control stubble segments after 2 months of incubation at 20°C (Table 7.2). *S. chartarum* caused more weight reduction than *Coprinus* sp., in terms of both fresh and dry weight.

Table 7.2. The effect of *Coprinus* sp. and *Stachybotrys chartarum* on weight reduction of canola stubble after 2 months of incubation at 20°C.

Test fungus	Mean decrease of stubble weight (mg)[1]	
	Fresh weight	Air-dry weight
Control[2]	5.1	12.1
Coprinus sp.	9.6	20.1
Stachybotrys chartarum	13.4	26.3

[1] Mean of the difference of weight of ten stubble segments from before inoculation to after incubation, fresh and air-dry weights.

[2] Each control stubble segment received 2 ml sterile distilled water, whereas each treated stubble segment was inoculated with two plugs of PDA culture of *Coprinus* sp. (plus 2 ml of sterile distilled water) or 2 ml of spore suspension of *S. chartarum*.

7.4 Discussion

Previous studies examined a range of bacteria (Kharbanda *et al.*, 1999; Zhao, 2001) or fungi (Hysek *et al.*, 2002; Karbanda & Dahiya, 1990; Maksymiak & Hall, 2000) with activity antagonistic to *L. maculans* on culture media or on canola plants. To our knowledge, the

present study provides new information on interactions between *L. maculans* and a wide range of fungal species, isolated from canola stubble or field soil, tested on agar media and on canola stubble. Some of these fungal species are known antagonists, decomposers or saprophytes. The present study also provides new information on the effects of these fungi on pseudothecium formation on canola stubble and on ascospore germination.

Macroscopic and microscopic observations, on PDA plates and agar-coated slides, respectively, showed a number of antagonistic interactions, including lysis, deformation, overgrowing and growth inhibition of *L. maculans* hyphae by *F. equiseti*, *G. roseum*, *T. aureoviride*, *Sordaria* sp. and the unknown Coelomycete. Inhibition of colony growth of *L. maculans* suggests that the *Fusarium* spp. tested may have produced diffusible metabolites that affected hyphae of *L. maculans*. In particular, deformation and lack of sporulation of *L. maculans* in the presence of *F. lateritium* and *F. sambucinum* might be related to such metabolites. See Addendum 6 Likewise, lysis and deformation of *L. maculans* hyphae on agar-coated slides in the presence of *G. roseum*, *Myrothecium* sp. and *T. aureoviride* indicates antibiosis. Also, *L. maculans* may be sensitive to antagonistic influences of, or be out-competed by, the saprophytic ability of other species tested, such as the unknown Coelomycete, *Arthrobotrys* sp. and *Monacrosporium* sp. The two latter fungi have shown antagonistic activity and decomposition of plant material (Cook, 1963).

The types of interactions between *L. maculans* and a number of the test species appeared to differ on PDA compared with water agar-coated slides, possibly due to differences in composition of the culture media or the culture environment. Further investigation of potential antagonists should include both macroscopic observation of colony morphology and microscopic observation of hyphal interactions. Co-inoculation of stubble with *L. maculans*

and the test species is another component of screening of potential antagonists, to evaluate their potential for reducing inoculum production of *L. maculans*. Further research is required to determine the mode of action of antagonists, to identify any inhibitory compounds responsible for antagonism and to determine the efficiency of antagonism on plant material.

Results support earlier findings (Blenis & Chow, 2005) that wood or canola stubble-associated fungi, including *Coprinus* spp., failed to eliminate *L. maculans* from nonsterile canola stubble either in the laboratory or in the field. In the present study, the pathogen was not eliminated from infested stubble that had been incubated at 15°C for 4-5 weeks. However, the reduction of pseudothecium density on naturally blackleg-affected stubble inoculated with *F. equiseti*, *G. roseum*, *T. aureoviride*, *Sordaria* sp. and the unknown Coelomycete to one-third or less than the density on the control stubble indicates antagonistic activity of these species against the blackleg pathogen. Further research, over extended periods of time, is required to examine the ability of these and other saprophytes and potential antagonists to colonise and displace the pathogen from infested residue. Such antagonism may have contributed to the reduction of *L. maculans* to negligible amounts on residues in soil 3 or more years after canola had been grown in South Australia (Sosnowski *et al.*, 2006). The method reported here might be useful for screening fungi isolated from different materials for their antagonistic activity against *L. maculans* on stubble in the laboratory. Promising antagonists would then need to be examined for efficiency using stubble in the field. In particular, the effects of environmental factors, such as temperature and moisture, on efficiency of activity of antagonists should be considered.

Blenis & Chow (2005) observed no significant decomposition of nonsterile canola stubble by wood or canola stubble-associated fungi in laboratory and field conditions. In contrast,

Cyathus olla was able to accelerate decomposition of canola stubble *in vitro*, a finding that needs to be confirmed under natural conditions as a means of reducing the incidence of stubble-borne diseases (Shinners & Tewari, 2000). Likewise, in this study *Stachybotrys chartarum* and *Coprinus* sp. reduced the weight of stubble compared with the control in the laboratory. The ability of *Coprinus* spp. to metabolise cellulose and lignin (Redhead & Traquair, 1981), and to antagonise some fungi (Ershova *et al.*, 2001), may have enabled the fungus to reduce stubble weight, decrease pseudothecium production on stubble and overgrow *L. maculans* on agar media. Although growth of *S. chartarum* was restricted on agar media, the fungus sporulated abundantly on colonized stubble segments. Stubble artificially inoculated with *S. chartarum* underwent more than twice the weight reduction of control stubble. Ability to degrade organic materials such as cellulose and wood, and antagonistic activity against numerous fungi (Domsch *et al.*, 1980) could explain the above-mentioned observations. *S. chartarum* has been reported to produce metabolites which inhibit a wide range of fungi, even other antagonists including *Myrothecium* sp. and *Trichoderma viride*, in culture media (Butt & Gaffar, 1972). Therefore, *Coprinus* sp. and *S. chartarum*, as effective decomposers of canola stubble and antagonists of *L. maculans*, may reduce pathogen inoculum in the field. This warrants further investigation, including longer-term field studies, to assess the suitability of these antagonists as a biological means of controlling blackleg.

In summary, growth and sporulation of *L. maculans* in culture media and on canola stubble was affected by a variety of fungi obtained from stubble buried in soil or from field soil. Results provide preliminary evidence of the antagonistic and competitive activities of some of the fungi tested. The role of stubble-associated fungi, individually and in combination, in biological control of *L. maculans* and decomposition of stubble warrants further study. The identification and deployment of biological agents to reduce inoculum of the pathogen may

reduce the need for fungicide application and contribute to more effective management of blackleg disease in the field.

Chapter 8. General discussion

This research provided new information about factors affecting the epidemiology of blackleg on canola in south-eastern Australia. The optimum temperature for pseudothecium maturation was 15°C. The optimum temperature of the range tested for germination of ascospores on detached cotyledons and leaves was 15-20°C, and 20°C was optimum for elongation of germ tubes, incubation period and number of ascospores released from stubble. Temperature had a greater influence on ascospore germination and elongation of germ tubes than did plant organ (cotyledon and leaf) and cultivar. Incubation period decreased with increasing leaf age, regardless of the blackleg-resistance rating of the cultivars. Darkness reduced pseudothecium formation on stubble compared with a 12 h photoperiod, and interrupted wetness increased the time taken for pseudothecia to mature. Burial of infested canola stubble in soil (in pots) affected pathogen survival, the population of stubble-associated fungi, pseudothecium development and stubble decomposition over 13 months in ambient conditions. Populations of fungi and bacteria isolated from soil on SEA were markedly greater than those on PRA and on CVA, respectively, over the 13 months. Growth and sporulation of *L. maculans* on agar media and on infested canola stubble in the laboratory were reduced due to antagonistic activities of a number of fungi obtained from stubble buried in soil. This research introduced a modified scale for pseudothecium development on stubble and a rapid method for screening of potential biological control agents.

Pseudothecia were not formed on dry stubble even when sprayed with water once a day, and mature pseudothecia discharged ascospores only after moistening of stubble at least twice daily. Therefore, the presence of moisture was an important factor affecting inoculum production in the controlled environment. This supports earlier field studies in south-eastern Australia, where ascospore discharge occurred significantly more in regions with 570 than

480 mm/year rainfall and more in 480 than in 375 mm/year rainfall (Marcroft *et al.*, 2003a). Likewise elsewhere, rainfall (Guo & Fernando, 2005; Huang *et al.*, 2005; Kruse & Verreet, 2005) and high relative humidity are considered crucial in ascospore discharge and, consequently, in the development of disease forecasting models (Gladders & Symonds, 1995; Pérès & Poisson, 1997; Pérès *et al.*, 1999; Salam *et al.*, 2003b). Knowing that continuous wetness for 22 and 28 days, at 15°C in a 12 h photoperiod, were appropriate conditions for pseudothecium formation and ascospore release, respectively, in the laboratory may facilitate estimation of the timing of maximum inoculum production in the field. If such findings are supported by field studies, this may enable us to optimize sowing time and timing of fungicide application to minimize exposure of susceptible crops to inoculum. Effective deployment of cultural and chemical controls of blackleg may reduce the risk of breakdown of resistance to *L. maculans* in canola, as maintaining durability of resistance genes depends on reducing the amount of inoculum, inoculum dispersal and pathogen population size (Aubertot *et al.*, 2006).

This project confirmed that temperature, wetness duration and cultivar affected germination of ascospores, hyphal growth and incubation period. Furthermore, plant organ (cotyledon and leaf) or leaf position affected ascospore germination and incubation period, respectively. Improved understanding of optimum conditions for germination of ascospores, hyphal growth and lesion development is important to optimize the timing of application of fungicides, such that plants are protected from infection when it is most likely to occur. Cultivar light leaf spot-resistance rating was used as one of several factors, including temperature, rainfall and disease incidence in the previous season, in the development of a model to predict the risk of light leaf spot caused by *Pyrenopeziza brassicae* on canola in the UK (Welham *et al.*, 2004). Currently, selection for resistance to blackleg on canola focuses on the stem canker phase.

However, leaf resistance may reduce selection pressure for the pathogen to overcome resistance that operates in the stem. Investigation of mechanisms responsible for the effects of cultivar, plant organ or leaf age on germination of ascospores, hyphal growth and incubation period could provide more information about potential defence mechanisms operating in the leaf, which might, in future, contribute to the development of new cultivars. Complete sequences of the genomes of *L. maculans* and *B. rapa*, which will be available soon (Fitt *et al.*, 2006b), will provide valuable information and new opportunities to study pre-penetration and post-penetration interactions between the pathogen and the host. Furthermore, a better understanding of the biology of the pathogen provides information helpful to select cultivars with stable and durable resistance genes (Howlett, 2004).

As leaf position was shown here to affect the incubation period of *L. maculans*, leaves at the same position should be used in future investigations of factors such as ascospore germination, penetration and incubation period in order to improve the uniformity of results. However, this recommendation was not applied in this project, in that the study of germination and hyphal growth from ascospores on first and second leaves of plants was conducted prior to the experiment concerning incubation period.

This research revealed that the time taken for pseudothecia to mature (after moist incubation) increased following burial. Due to time constraints, stubble placed on the soil surface was not included as a control in studies of the effect of burial on decline of *L. maculans* and inoculum production. The time taken for pseudothecia to mature was 22 days on stubble that was not buried (see chapter 3) but 30 days on stubble retrieved from soil after 10 months (see chapter 6). Therefore, burial of stubble by ploughing may increase the time required for pseudothecia to develop and mature, if the buried stubble is brought to the soil surface again, compared

with stubble left on the soil surface after harvest. Furthermore, increasing the time taken for pseudothecia to mature means that approximately 8 more days of favourable weather conditions may be required for production and discharge of ascospores. This research also revealed that inoculum production on stubble was reduced when *L. maculans* declined on stubble buried in soil. The density of pseudothecia formed on unburied stubble, 53.4 per 0.5 × 1 cm, compared with 1.9 per unit area of stubble after 10 months of burial in field soil, shows that burying stubble may decrease inoculum production. Although storage of stubble (for 5 months in the laboratory) retrieved from sand and field soil in this research did not affect the density of pseudothecia formed on stubble after moist incubation, Marcroft *et al.* (2003a) reported a decrease of ascospore discharge with ageing of stubble in the field. Pseudothecia from 6-month-old stubble left on the soil surface discharged 30-fold as many ascospores per hectare of the field as older stubble (18-42 months old) in Victoria (Marcroft *et al.*, 2003a). However, 42-month-old stubble was still able to discharge ascospores. Marcroft *et al.* (2003a) also reported that burning of stubble and use of resistant cultivars (Marcroft *et al.*, 2004) reduced ascospore release, but did not stop it. In contrast, burying of stubble in the present study resulted in cessation of inoculum production after 11 months. This comparison suggests that inoculum declines more quickly on buried stubble than on unburied or burnt stubble. Although *L. maculans* survived on stubble after 13 months, inoculum production was not detected. Furthermore, *L. maculans* declined to undetectable levels on residues in soil 3 or more years after cropping in South Australia (Sosnowski *et al.*, 2006). Therefore, farmers should, wherever possible, bury canola stubble soon after harvest to reduce inoculum production in their fields.

The optimum temperature of the range tested for the number of ascospores released, hyphal growth and incubation period in this study was 20°C, however, more pseudothecia and

ascospores developed earlier at 15°C. The longer maturation time and lesser pseudothecium density at 20°C may be due to microbial activity on stubble that was favoured at 20°C compared to 15°C. This suggestion is supported by earlier findings (Blenis & Chow, 2005) that all but one of 55 isolates from wood and canola stubble showed up to 70% growth reduction on agar at 13°C, relative to growth at 22°C. Various stubble-associated fungi, such as *Fusarium equiseti*, *Gliocladium roseum*, *Trichoderma aureoviride*, *Sordaria* sp. and an unknown Coelomycete, reduced pseudothecium density and sporulation of *L. maculans* in this research, suggesting that *L. maculans* competed poorly with those fungi on stubble. The effect of temperature on the interactions between *L. maculans* and these fungi should be examined *in vitro* and *in planta* to test this suggestion.

The combination of activities, such as antagonism, parasitism and competition, of the stubble-associated fungi and fungi isolated from soil may have contributed to the decreasing isolation frequency of *L. maculans* over time. *Stachybotrys chartarum* and *Coprinus* sp., both of which were frequently isolated from stubble buried in soil, decomposed blackleg-free stubble, significantly reduced the density of pseudothecia when inoculated onto naturally blackleg-affected stubble and antagonized *L. maculans* in dual culture on agar. The ability of these two fungi to antagonize other fungi and decompose plant material has been documented previously (Domsch *et al.*, 1980; Ershova *et al.*, 2001; Redhead & Traquair, 1981). Also, some *Fusarium* spp., commonly isolated from stubble buried in soil, antagonized *L. maculans* on agar and *F. equiseti* reduced the density of pseudothecia on naturally blackleg-affected stubble in the laboratory. Thus, these fungi have potential to reduce inoculum and survival of *L. maculans* on stubble in the field. This suggestion requires experimental confirmation under ambient conditions. Although the unknown Coelomycete was isolated less frequently from stubble than the fungi mentioned above, it showed the best antagonistic activity against *L.*

maculans in the laboratory. Therefore, the unknown species also has potential as a means of reducing inoculum, provided that it can be identified and shown to be non-pathogenic to canola and other crops.

Arthrobotrys sp. and *Monacrosporium* sp., mycoparasites able to partially decompose plant material (Domsch *et al.*, 1980), and *Gliocladium roseum*, *Trichoderma aureoviride*, *Sordaria* sp., *Myrothecium* sp. and *Penicillium* sp. antagonized *L. maculans* on culture media and reduced pseudothecium development on stubble. However, these antagonists did not establish on buried stubble as well as the species mentioned above, perhaps due to inhibitory effects of soil characteristics, sensitivity to soil moisture content (Kouyeas, 1964) or inability to compete with other decomposers in field soil. Thus, their potential as biological agents for reducing inoculum appears limited but may warrant further investigation.

As stated above, among the antagonists and decomposers studied here, *S. chartarum*, *Fusarium* spp. and *Coprinus* sp. appeared to have the best potential to reduce stubble-borne inoculum of *L. maculans*. However, the role of soil microorganisms should be considered also, as some fungi isolated from the stubble differed in frequency of isolation from soil and sand and, also, a number of stubble-associated species were isolated from soil adjacent to buried stubble. In addition, all the above-mentioned activities are likely to be affected by a variety of environmental factors in the soil and in air. Suboptimal soil moisture and soil temperature are known major physical constraints to biodegradation of crop residues (Steiner *et al.*, 1999). Future research could improve the understanding of this complex combination of fungal activities on stubble buried in a range of types of soil and environmental conditions. Furthermore, pre-treatment of canola leaves with ascospores of *L. biglobosa* delayed lesion appearance, and reduced lesion area and blackleg severity on plants inoculated with *L.*

maculans, suggesting that pre-treatment with biological agents can stimulate local and systemic resistance to blackleg in canola (Liu *et al.*, 2006). Further research should be undertaken to investigate the effects of the various antagonists examined in this project on defence mechanisms in canola. A better understanding of the interactions between *L. maculans* and associated fungi on canola stubble and on foliage could contribute to integrated disease management.

In conclusion, ascospore germination, hyphal growth, infection of cotyledon and leaf, pseudothecium and ascospore development and release were influenced more by wetness rather than by temperature in the controlled environment in the present investigation. Thus, lack of moisture might be the most limiting factor in nature in comparison with temperature, light, cultivar and plant tissue. In the presence of appropriate wetness, 20°C was the optimum temperature of the range tested for infection of leaf, inoculum production and discharge from stubble. Knowledge of the time required for inoculum production and disease development in a range of conditions in the laboratory gives an estimate of the number of days with favourable conditions required for blackleg development in the field. The effects of other environmental factors and other temperatures on the disease cycle require further investigation. Controlled-environment studies allow the influence of each factor to be examined individually, whereas in nature, a complicated combination of known and unknown factors affects the outcome. Nevertheless, findings on the etiology of *L. maculans* in the laboratory need further investigation in the field. The acquisition of such biological and climatic data should ultimately allow a climate-based disease forecast system to be developed, as an aid to improving management of blackleg in south-eastern Australia.

See Addendum 7.

Appendix 1.

Summarized code for stages of development in canola (*Brassica napus* L.) developed by Sylvester-Bradley (1985).

Definition	Code
Germination and emergence	0
Leaf production	1
Both cotyledons unfolded and green	1,0
First true leaf	1,1
Second true leaf	1,2
Third true leaf	1,3
Fourth true leaf	1,4
Fifth true leaf	1,5
About tenth true leaf	1,10
About fifteenth true leaf	1,15
Stem extension	2
No internodes ("rosette")	2,0
About five internodes	2,5
Flower bud development	3
Only leaf buds present	3,0
Flower buds present but enclosed by leaves	3,1
Flower buds visible from above ("green bud")	3,3
Flower buds level with leaves	3,4
Flower buds raised above leaves	3,5
First flower stalks extending	3,6
First flower buds yellow ("yellow bud")	3,7
Flowering	4
First flower opened	4,0
50% all buds opened	4,5
Pod development	5
30% potential pods	5,3
All potential pods	5,9
Seed development	6
Seeds expanding	6,1
All seeds black and hard	6,9
Leaf senescence	7
Stem senescence	8
Most stem green	8,1
Little stem green	8,9
Pod senescence	9
Most pods green	9,1
Few pods green	9,9

Appendix 2.

Culture media used in experiments were as follows:

Crystal violet agar (CVA)

Neutralised bacteriological peptone (Oxoid)	5 g
Beef extract (Bovril)	5 g
Agar (Bacto™ Agar, Difco)	20 g
RO water	1000 ml

Add 2 ppm crystal violet (Merck) immediately before pouring agar.

Dilute V-8 Agar (DV8A)

Diluted V-8 juice*	20 ml
$CaCO_3$	0.2 g
Agar (Bacto™ Agar, Difco)	17 g
RO water	980 ml
Streptomycin sulphate (Sigma S0890)	30-100 mg

* V-8 juice (Erwin *et al.*, 1987) was cleared by filtration through single then double layers of filter paper (Whatman, no. 41).

Nutrient agar (NA)

Nutrient broth (Oxoid)	13 g
Agar (Bacto™ Agar, Difco)	20 g
RO water	1000 ml

Peptone-rose Bengal agar (PRA)

Neutralised bacteriological peptone (Oxoid)	5 g
Dextrose	10 g
KH_2PO_4	1 g
$MgSO_4.7H_2O$	0.5 g
Rose Bengal (Merck)	30 mg
Agar (Bacto™ Agar, Difco)	20 g
RO water	1000 ml
Streptomycin sulphate (Sigma S0890)	30-100 mg

Potato dextrose agar (PDA)

Potato dextrose agar (Difco)	39 g
RO Water	1000 ml
Streptomycin sulphate (Sigma S0890)	30-100 mg

Soil-extract agar (SEA)

Soil extract	1000 ml
K_2HPO_4	0.2 g
Agar (Bacto™ Agar, Difco)	15 g

To prepare soil extract, 500 g fertile soil (Waite Campus, Adelaide, South Australia) in an amount of RO water, which yielded 1000 ml of extract, was autoclaved. After cooling, the

extract was filtered through single layer of filter paper (Whatman, no. 41) and the cloudy filtrate was refiltered. The medium was adjusted to pH 6.8 and autoclaved (James 1958).

Water agar

Agar (Bacto™ Agar, Difco)	20 g
RO water	1000 ml
Streptomycin sulphate (Sigma S0890)	30-100 mg

Culture media were autoclaved at 121°C for 20 minutes and streptomycin sulphate was added, where required, to molten media, once cooled to approximately 45°C. Agar media were dispensed into 9 cm diameter Petri dishes or poured into tubes and allowed to cool and solidify.

Appendix 3.

Mean percent germination of *Leptosphaeria maculans* ascospores from three repetitions of the experiment on agar-coated slide, detached cotyledon and leaf of five canola cultivars after 2-24 h of incubation time at 5, 10, 15 & 20°C.

Time	Substrate	Temperature (°C)							
(h)		5		10		15		20	
		C	L	C	L	C	L	C	L
2	Hyola 60	0[1]	0	9	7	25	21	37	31
	Ripper	0	0	12	6	21	19	39	30
	Monty	0	0	12	8	29	25	39	33
	Karoo	0	0	12	10	26	25	38	31
	Q2	0	0	11	7	27	24	39	34
	Agar	0		10		28		40	
4	Hyola 60	2	0	31	26	62	52	65	62
	Ripper	1	0	31	18	53	49	62	62
	Monty	1	1	31	32	65	62	66	60
	Karoo	2	1	34	34	70	65	65	63
	Q2	3	2	38	33	70	66	66	61
	Agar	3		41		69		70	
6	Hyola 60	12	6	50	39	66	69	83	80
	Ripper	10	4	47	41	71	71	82	79
	Monty	12	9	48	47	78	74	81	79
	Karoo	13	10	49	51	79	76	85	80
	Q2	15	11	52	51	81	74	88	82
	Agar	15		50		76		85	
8	Hyola 60	20	17	59	50	82	80	88	80
	Ripper	23	13	61	53	81	81	88	80
	Monty	23	20	65	55	81	81	91	86
	Karoo	23	22	59	60	83	80	90	88
	Q2	30	22	68	67	82	81	91	89
	Agar	23		66		81		89	
10	Hyola 60	45	29	65	59	90	87	90	80

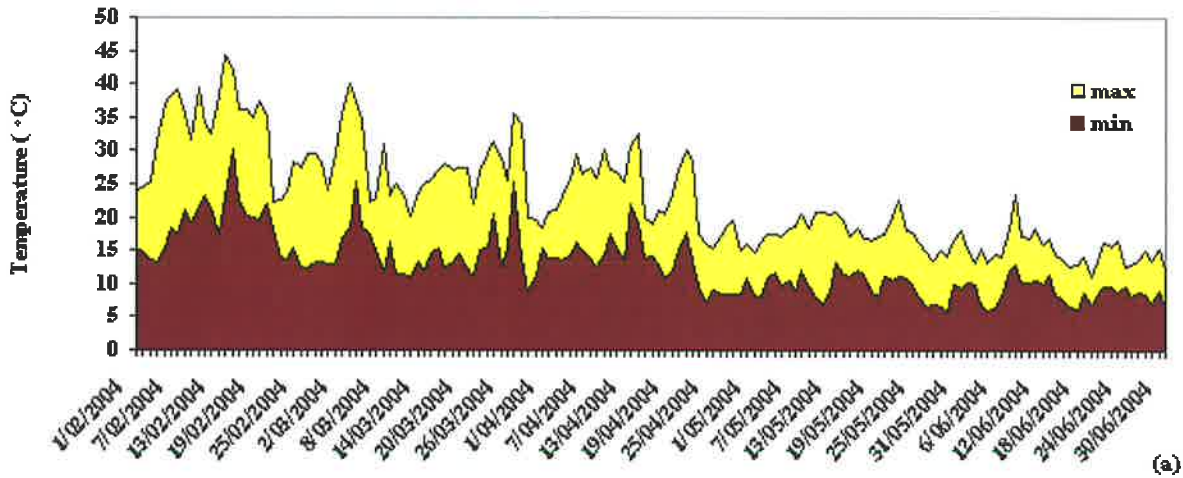
	Ripper	43	23	60	59	90	87	91	85
	Monty	44	35	69	65	90	89	89	86
	Karoo	48	33	71	71	90	88	90	88
	Q2	49	35	72	71	92	88	92	91
	Agar	46		71		88		91	
24	Hyola 60	81	80	81	79	90	90	90	88
	Ripper	81	76	84	78	90	90	91	87
	Monty	83	82	82	80	91	90	91	91
	Karoo	83	82	85	84	91	90	92	88
	Q2	83	81	87	84	92	90	93	92
	Agar	80		86		91		92	

C = detached cotyledon; L = detached leaf.

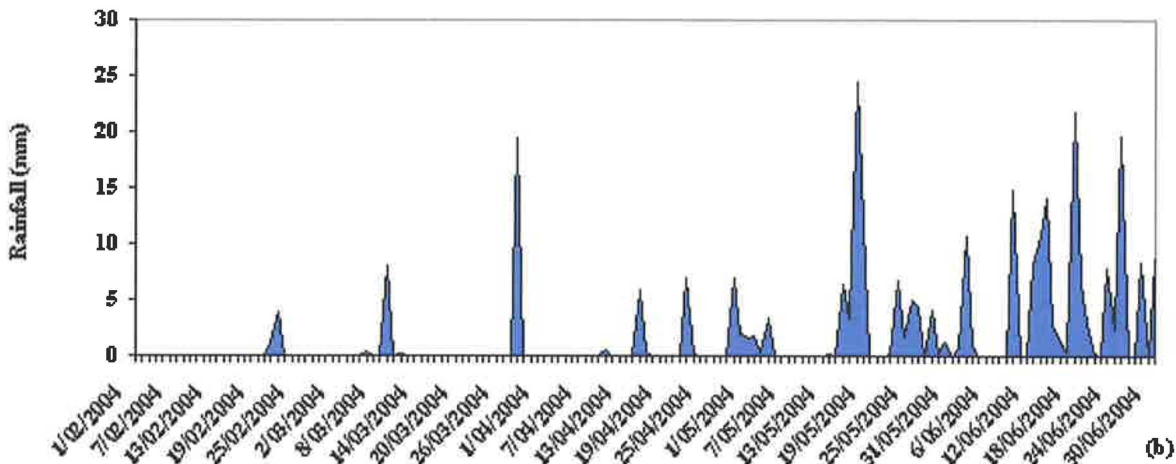
[1] LSD = 9; $P > 0.05$.

Appendix 4.

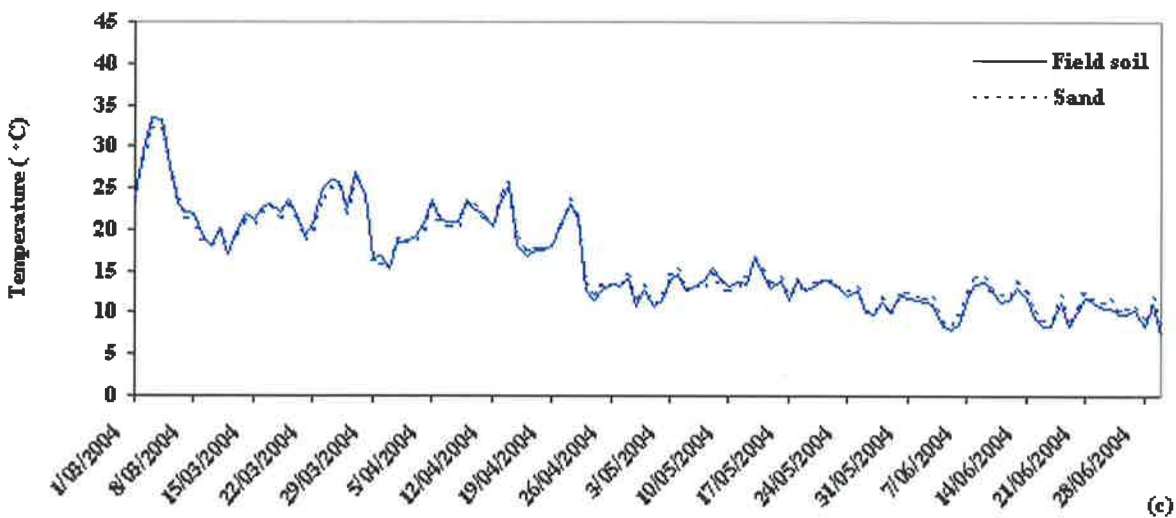
Environmental data collected at Waite Campus; (a-b) temperature and rainfall collected by a weather station (Model T metstation), (c) soil temperature inside sand and field soil pot collected using Hastings data loggers.



(a)

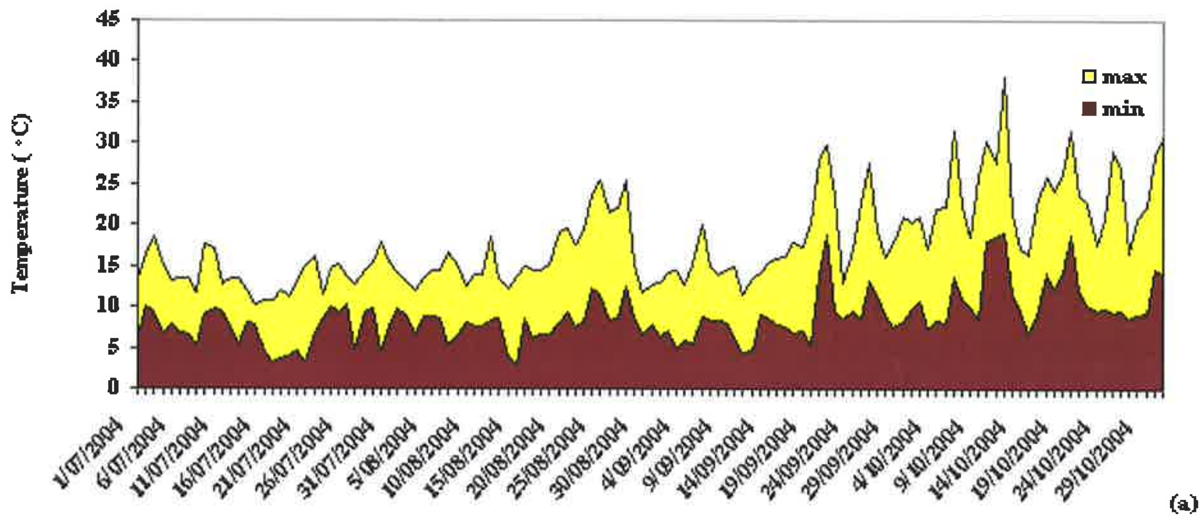


(b)

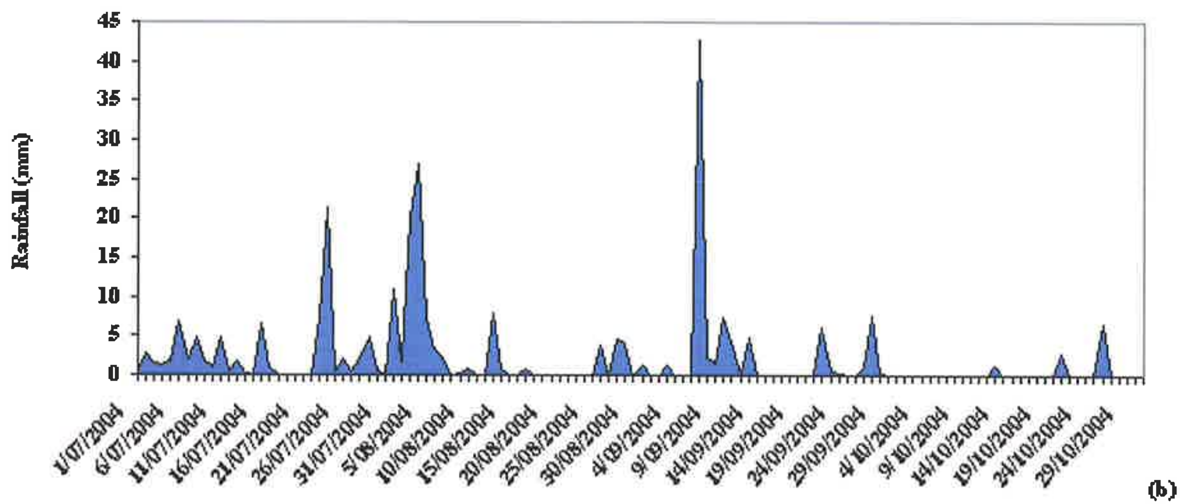


(c)

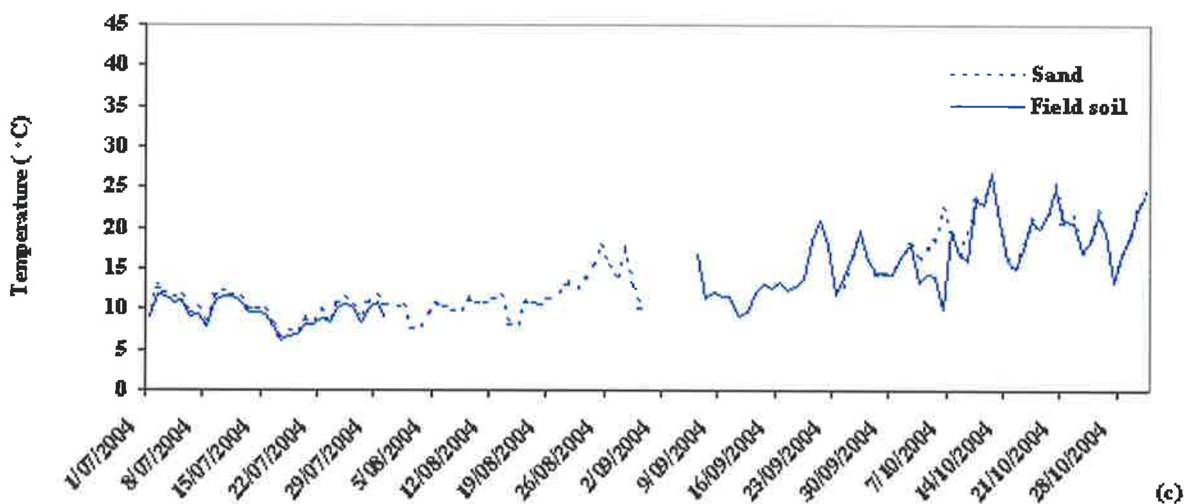
Appendix 4. continued



(a)

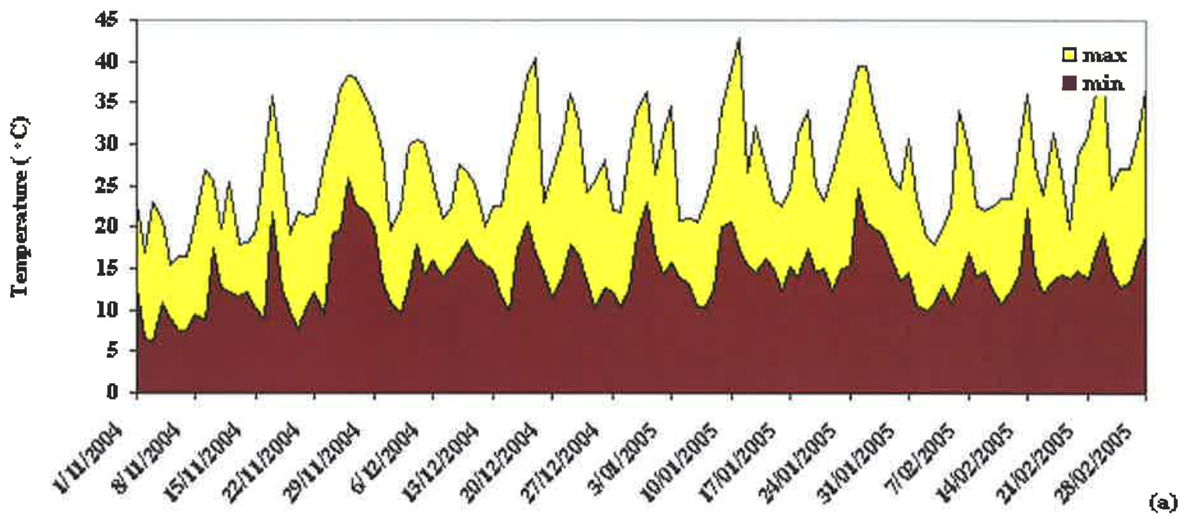


(b)

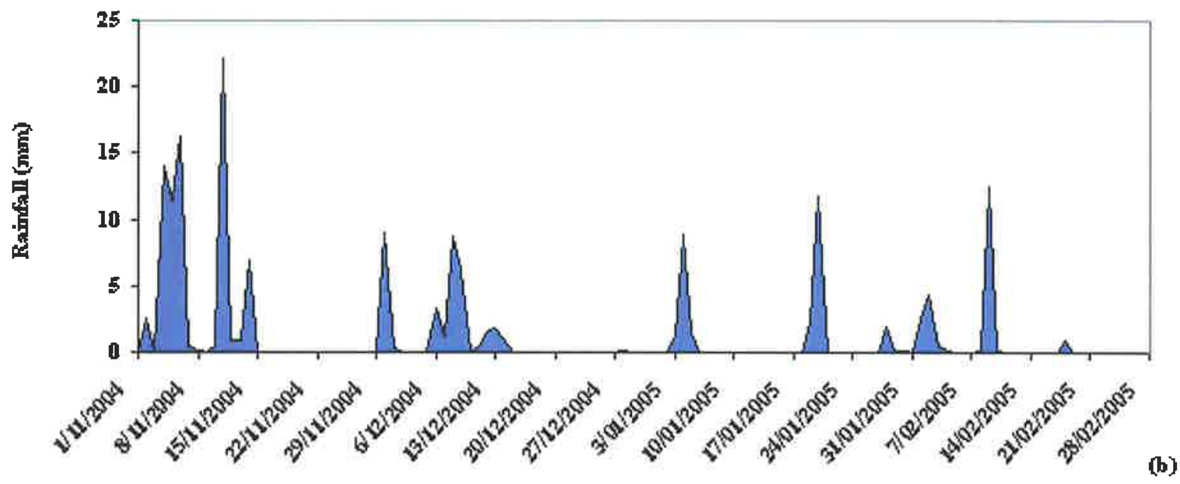


(c)

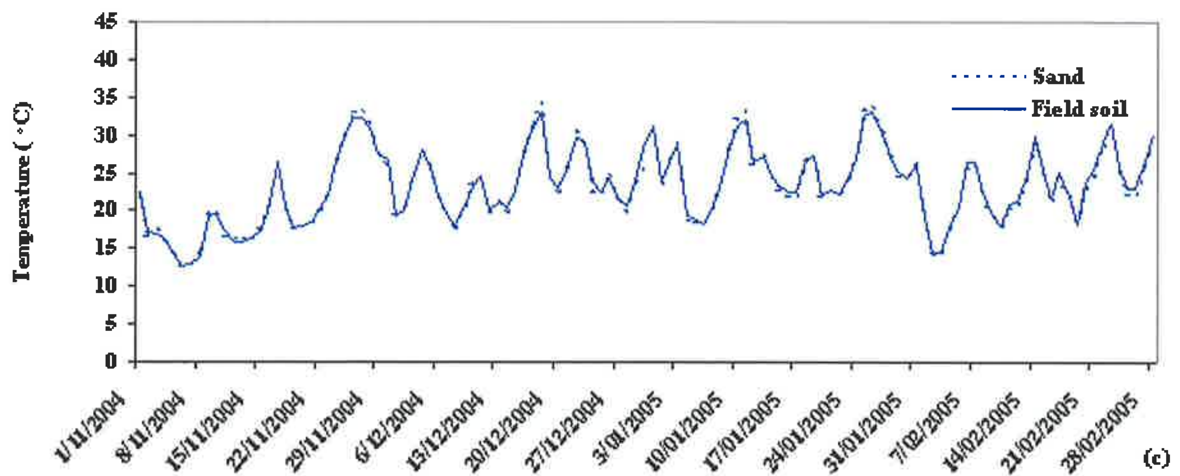
Appendix 4. continued



(a)



(b)



(c)

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Addenda

1. Page 12, 1.3.2.3, line 4. Salam *et al.* (2003b) reported that ascospore release in Western Australia began at an hourly rainfall of >2 mm and the distance travelled by ascospores was dependent on wind speed.

2. Page 78, paragraph 2, line 4. As germination and hyphal growth on leaves were compared with those on cotyledons (chapter 4), this study focused on incubation period for leaves at different positions.

3. Page 92, end of paragraph 1. In addition, shorter incubation period following longer wetness durations at 10 and 20ºC suggests that longer availability of wetness increased germination and hyphal growth from ascospores on the leaf surface, resulting in quicker infection of more stomata than that following 16 h of wetness. This is supported by earlier findings (Huang *et al.*, 2003b) in the UK, that temperature and time after inoculation of the leaf affected the percentage of stomata penetrated by germ tubes from *L. maculans* ascospores.

4. Page 95, paragraph 1. Although single-gene race-specific resistance in Hyola 60 has been overcome by isolates of *L. maculans* in some regions of Australia (Sprague *et al.*, 2006), leaves of this cv. might have provided an inhibitory substance which reacted with the pathogen in a manner different from the other cultivars tested.

5. Page 134, after paragraph 1. Greater moisture content of soil in pots due to rainfall over May-November may also explain why fungi such as *Arthrobotrys* sp., *Coprinus* sp., *Gliocladium roseum*, *Monacrosporium* sp., *Rhizoctonia* sp., *Trichoderma* spp. and the unknown Coelomycete from buried stubble were isolated frequently following rain. It is

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known that rewetting soil increases the activity of microbial decomposers (Van Veen & Kuikman, 1990). Therefore, rainfall frequency and soil moisture regime could have influenced the colonization of stubble by the above-mentioned fungi in the present study. Therefore, burying of stubble in soil during wetter months may result in greater decomposition.

6. Page 151, paragraph 2. Formation of the zone of inhibition between *L. maculans* and *Fusarium acuminatum*, and also formation of the zone of sparse hyphae in the colony of *F. lateritium* in the presence of *L. maculans* may be due to secretion of a toxin, such as Sirodesmin PL, by *L. maculans*.

7. Page 161, last paragraph. The data on the effects of environmental conditions on ascospore germination, hyphal growth, incubation period, pseudothecium maturation and ascospore release, obtained in the present research, could be integrated to develop a simple model to estimate the timing of conditions most appropriate for infection of canola. To develop the model, further analysis of data is needed to obtain an appropriate equation which deals with all these factors together (D. Shtienberg, pers. com., 2005). However, such a model would need to include more environmental factors and to be evaluated in South Australian fields to be validated.