Photograph taken by R. Kimber

STUDIES ON THE EPIDEMIOLOGY OF BLACKLEG (*LEPTOSPHAERIA MACULANS*) AND MECHANISMS OF RESISTANCE IN CANOLA.

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Thesis submitted for the degree of

Doctor of Philosophy

in

The University of Adelaide

Department of Applied and Molecular Ecology

Faculty of Sciences

December 2002

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ABSTRACT

Blackleg disease of canola (*Brassica napus*) is caused by the fungus *Leptosphaeria maculans*. It has the potential to reduce crop yield by up to 80% in a severe epidemic. In this project, three aspects of the disease were examined.

Mechanisms of resistance in Australian canola cultivars were investigated using scanning electron microscopy (SEM) and histochemical stains in combination with light microscopy. SEM suggested that the major mechanism of stem resistance was located in the junction of the petiole and stem of plants and that the cultivar Hyola 60 had the ability to resist invasion by *L. maculans* at the leaf surface. Histochemical stains showed that lignin and suberin were produced in the epidermal cell walls and guard cells surrounding stomata in both resistant and susceptible cultivars in response to infection with *L. maculans*. It appeared that resistant cultivars had higher concentrations of both compounds than the susceptible cultivars.

The epidemiology of the disease was studied, both in the field and in controlled conditions. In the field, most cultivars that showed resistance to stem and cotyledon infection were susceptible to leaf infection. Infection by ascospore inoculum was favoured by periods of increased rainfall, temperature and wind activity and infection was most prevalent early in the season, when plants were most vulnerable to leaf infection. Controlled environment experiments revealed that the optimal conditions for stem infection were a temperature regime of 23°C with at least 48 h of leaf wetness, at least 10^6 pycnidiospores/ml water and infection of leaves up until the three-leaf stage.

A DNA-based method of detection of *L. maculans*, which was developed by the Root Disease Testing Service, South Australian Research and Development Institute using *L. maculans* specific primers acquired from the CSIRO, was validated for quantifying DNA in stubble and

soil samples. A selective medium was developed to enumerate colonies of *L. maculans* from infected canola stubble and a plant bioassay was developed to estimate the disease potential of infected stubble. Milled and ground canola stubble was mixed with oaten hay (*Avena sativa* L.) in a series of fractions and spread on plates of the selective medium and over plants in a bioassay. There was a strong correlation ($R^2 > 0.94$) between these results and the estimated amount of *L. maculans* DNA. After validation, the procedure was used to quantify *L. maculans* DNA in soil samples; which revealed that levels decreased as stubble decomposed and that *L. maculans* could not be detected in fields in which canola had been grown more than 2 years earlier.

Finally, during this study *L. maculans* was observed to have colonised the roots of canola plants in the field. Canola seedlings artificially inoculated via the roots or hypocotyls developed root infection and crown canker. It was concluded that *L. maculans* was able to infect the roots of canola via the stem or directly from the soil.

DECLARATION

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

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

7th May 2003

Signed Date

ACKNOWLEDGEMENTS

I would like sincerely to thank my supervisors, Dr. Eileen Scott and Mr. Mark Ramsey for their guidance, support, advice and many hours of thesis editing. I am particularly grateful for their confidence in my ability and encouragement throughout this project.

For financial support, I wish to acknowledge the Australian Research Council (ARC), the South Australian Grains Industry Trust (SAGIT) and the South Australian Research and Development Institute (SARDI).

I would also like to thank;

Mr Rohan Kimber for scientific and technical support and assistance as well as his moral support and friendship.

Ms Jenny Davidson for advice, scientific and technical support and supplying soil samples collected from around South Australia.

All my colleagues in the Field Crops Pathology Unit, SARDI, especially Dr Alan Dubé who retired in November 2001, for their support and assistance.

Dr Eileen Scott's fungal pathology research group for their friendship, advice and constuctive criticism during seminar practices.

Mr Trent Potter (SARDI) for assisting with agronomic advice and supplying canola seed and stubble samples.

Dr Herdina, Dr Kathy Ophel-Keller, Dr Alan McKay and Mr Russel Burns (Root Disease Testing Service, SARDI) for processing soil and stubble samples, conducting DNA-based assays, and for their assistance and advice.

Dr Marilyn Henderson, Ms Linda Olssen and all the staff at CEMMSA, University of Adelaide, for training and assistance on the scanning electron microscope.

Dr Meredith Wallwork (University of Adelaide) for instruction in histochemical methods and providing stains.

Mr Neil Wratten (New South Wales Agriculture) for supplying doubled haploid canola seed.

Dr Gordon Murray (New South Wales Agriculture) for supplying isolates from canola roots and assistance with publishing a new disease report.

To all those the many people who have given assistance and advice during this project; Mr Steve Marcroft (Agriculture Victoria), Dr Martin Barbetti (Department of Agriculture, Western Australia), Dr Barbara Howlett (The University of Melbourne), Mr Dennis Ballinger (Dovuro) and Mr Mark Stanley (Primary Industries and Resources South Australia).

Mr Andrew Easton (Pacific Seeds) for supplying Hyola 60 seed.

For their administrative support; Ms Susan Eaton, Mr Terry Feckner, Ms Heather Fraser, Mr Gary Taylor (Dept. Applied and Molecular Ecology), Ms Tracy O'Malley and Ms Annette Hutson (SARDI Field Crops Pathology).

Mr Michael Elleway (University of Adelaide) and Mr Peter McCormack (SARDI) for preparing field trial sites on the Charlick and Kingsford Research Stations, respectively.

Mr Rob Wheeler and Mr Larn McMurray (Field Crop Evaluation, SARDI) for sowing, maintaining and harvesting field trials.

Ms Christine Schutz and Mr David Sosnowski for technical assistance.

Dr Alexandra Schoeny (INRA, France) for instruction on the SAS statistical package and advice on modelling field trial data.

Mr Andreas Kiermeier for statistical advice.

Mr Ernie Nagy, Mr Paul Ingram and Mr Reg Skipworth for glasshouse support and maintenance.

John and Ange Rohde for allowing me to sample fields on their property near Tarlee in South Australia.

To my family and friends for all their support and encouragement throughout this project, especially my wife, Mary.

PUBLICATIONS AND CONFERENCE PROCEEDINGS

Scientific Publications

Sosnowski, M., Ramsey, M., Murray, G., Scott, E. and Wilmshurst. C. (2001). Symptoms of blackleg (*Leptosphaeria maculans*) on the roots of canola in Australia. *Plant Pathology* **50**: 808 [http://www.bspp.org.uk/ndr/jul2001/2001-34.htm]

CAB International, 2003. *Leptosphaeria maculans* [reviewed by M Sosnowski]. In: Crop Protection Compendium, 2003 edition. Wallingford, UK: CAB International.

Marcroft, S.J., Sosnowski, M.R., Scott, E.S., Ramsey, M.D., Salisbury, P.A. and Howlett, B.J. (2003). Relationship between the growth stage of *Brassica napus* (canola) and the severity of *Leptosphaeria maculans* (blackleg) infection. (in preparation for Plant Pathology)

Conference Proceedings

Sosnowski, M., Scott, E. and Ramsey, M. (2001). Detection and Survival of *Leptosphaeria maculans*. *12th Australian Research Assembly on Brassicas, Geelong, Australia, October 2001*. pp 223-226.

Sosnowski, M., Scott, E. and Ramsey, M. (2001). Detection and Survival of *Leptosphaeria maculans*. *13th Biennial Conference, Australasian Plant Pathology Society, Cairns, Australia, September 2001*. p 170.

Sosnowski, M., Scott, E. and Ramsey, M. (2001). Epidemiology of blackleg (*Leptosphaeria maculans*) in canola. *13th Biennial Conference, Australasian Plant Pathology Society, Cairns, Australia, September 2001*. p 385.

Sosnowski, M., Scott, E. and Ramsey, M. (2001). Investigating resistance and epidemiology of blackleg (*Leptosphaeria maculans*) in canola. *12th Australian Research Assembly on Brassicas, Geelong, Australia, October 2001*. pp 82-86.

Sosnowski, M. (2002). Canola disease research update. *2002 Cropping Systems Update, Adelaide, Australia February 2002*. pp 101-103. [http://www.grdc.com.au/growers/res_upd/south/02/RU_S02_ADEL_P47.htm]

Sosnowski, M.R., Scott, E.S. and Ramsey, M.D. (2003). Epidemiological studies of *Leptosphaeria maculans* on canola in a controlled environment. *8th International Congress of Plant Pathology, Christchurch, New Zealand, February 2003*. p 105.

Potter, M.J., Sosnowski, M.R., Scott, E.S. and Ramsey, M.D. (2003). Biochemical basis for canola leaf resistance to blackleg. *8th International Congress of Plant Pathology, Christchurch, New Zealand, February 2003*. p 196.

Sosnowski, M.R., Scott, E.S. and Ramsey, M.D. (2003). Epidemiological studies of *Leptosphaeria maculans* on canola in Australian field conditions. *11th International Rapeseed Congress, Copenhagen, Denmark, July 2003*. (accepted)

Sosnowski, M.R., Scott, E.S. and Ramsey, M.D. (2003). Epidemiological studies of *Leptosphaeria maculans* on canola in a controlled environment. *11th International Rapeseed Congress, Copenhagen, Denmark, July 2003*. (accepted)

ABBREVIATIONS

¼ PDA	potato dextrose agar (quarter strength)
ACAS	Australian Crop Accreditation System
AFLP	amplified fragment length polymorphism
AG	anastomosis groups
ANOVA	analysis of variance
AWS	automatic weather station
CAA	Canola Association of Australia
CEMMSA	Centre for Electron Microscopy and Microstructure Analysis
CFU	colony forming units
CPD	critical point drier
CSIRO	Commonwealth Scientific and Industrial Research Organisation
cv.	cultivar
cvs	cultivars
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
FESEM	field emission scanning electron microscope
GS	growth stage
ITS	internal transcribed spacer
MPN	most probable number
NSW	New South Wales
OM	organic matter
PBS	phosphate buffered saline
PCNB	pentachloronitrobenzene
PCR	polymerase chain reaction
PDA	potato dextrose agar (full strength)
RAPD	random amplified polymorphic DNA
RDTS	Root Disease Testing Service
RFLP	restriction fragment length polymorphism
RO	reverse osmosis
RS	research station
SA	South Australia
SARDI	South Australian Research and Development Institute
Vic.	Victoria
WA	Western Australia

CHAPTER 1

INTRODUCTION

Blackleg disease of canola (*Brassica napus* L. var. *oleifera* (Metzer) Sink.) is caused by the fungus *Leptosphaeria maculans* (Desm.) Ces. et de Not. [anamorph = *Phoma lingam* (Tode: Fr) Desm.]. It has the potential to devastate crops, as it did in Western Australia in 1972, where blackleg was the main reason for the reduction in the area of *B. napus* cultivars sown from 49,000 ha in that year to 2,000 ha in 1974 (Bokor *et al.* 1975). The area sown to canola in Australia has expanded six fold over the past decade, due to the development of cultivars better adapted to Australian conditions and the increased profitability of the crop on acid soils and in lower rainfall areas (Colton and Potter 1999). This expansion has resulted in increased cropping frequencies and, hence, has increased the risk of disease on the canola crop.

Management of blackleg is based on resistance, which reduces yield loss and decreases the inoculum potential for future crops. Some mechanisms of resistance have been identified in the leaves (Xi and Morall 1993; Chen and Howlett 1996; Sosnowski *et al.* 2001b) and stems (Hammond and Lewis 1987b; Nathaniels and Taylor 1983; Xi and Morall 1993; Annis and Goodwin 1997a; Chen and Seguin-Swartz 1999) of canola plants. However most of these studies were carried out on cultivars that are not grown in Australia currently. As new sources of resistance are continually being sought to combat the threat that this genetically diverse fungus will overcome resistance, it is important that new mechanisms of resistance are identified to assist breeders in incorporating them into current cultivars.

Environmental conditions such as the weather play a crucial role in the epidemiology of *L. maculans* (West *et al.* 2001). Information on the development of disease symptoms in a range of different weather conditions will aid in the prediction of epidemics and potential yield loss.

It is also important to determine the reaction of a range of cultivars to the disease in different climates to help growers select the best cultivar for their situation. Other factors such as inoculum concentration and plant maturity at inoculation may also have an effect on the epidemiology of the fungus and hence the management of the disease.

Several DNA-based techniques have been developed for the identification of *L. maculans* and for differentiation between A and B-group strains from pure culture (Goodwin and Annis 1991; Koch *et al.* 1991; Morales *et al.* 1993; Mahuku *et al.* 1997; Balesdent *et al.* 1998; Voigt *et al.* 1998; Purwantara *et al.* 2000; Sosnowski *et al.* 2001b), from plant tissue (Mahuku *et al.* 1996) and from ascospores collected in a spore trap (Calderon *et al.* 2002). There is a need for more robust assays that can be used for detection of *L. maculans* in stubble and soil. Further development of a DNA-based assay, by comparing it with conventional methods of disease detection such as plating on agar media and plant bioassays, would lead to an efficient and economical diagnostic test for soil and stubble samples.

The research described in this thesis was undertaken to i) examine the mechanisms of resistance of canola to *L. maculans*, ii) explore epidemiological aspects of the disease, iii) validate a DNA-based assay as a diagnostic tool and iv) investigate the survival of the pathogen.

CHAPTER 2

LITERATURE REVIEW

2.1 INTRODUCTION

Blackleg disease of canola, which is caused by the ascomycete fungus *L. maculans*, is known to infect all above ground parts of the plant. Ascospores infect leaves via stomata and wounds to cause leaf lesions from which hyphae grow down the petiole into the stem. The fungus causes stem lesions, which can eventually girdle the stem, restricting transport of nutrients and water to the pods and hence reducing yield (Hammond and Lewis 1987a; Chen and Howlett 1996; Davies 1986, cited by West *et al.* 1999). The fungus can survive on the stubble over the summer and infect crops in following seasons.

This review examines the pathogen, *L. maculans*, and its interactions with canola. It explores the history of canola from the origins of oilseed rape and the effect that blackleg has had on the industry. The epidemiology of this disease will be examined by looking at the symptoms in detail, the life cycle of *L. maculans*, the carry-over of inoculum and the levels of infection which occur. Control of blackleg can be achieved through a number of different management strategies. To manage blackleg, it is essential that we understand the variability in pathogenicity of this fungus and the mechanisms by which the host can resist it. The importance of further research on plant-pathogen interactions to the continuing success of the canola industry is demonstrated in this review.

2.2 BACKGROUND

2.2.1 History of canola and blackleg

Oilseed rape has been used as an agricultural crop for centuries in Europe and Asia. It was recorded in India as early as 2000 BC, introduced to Japan from China about 35 BC, and large

scale planting began in Europe in the 13th century (Downey *et al.* 1970; Lamont 1990). The word rape is derived from the Latin word rapum, which means turnip. Prior to its widespread cultivation, seed was gathered from wild forms of the plant and the oil extracted to burn for light and to produce soap (Gugel and Petrie 1992). During the 19th century, oilseed rape production increased throughout Europe and then during the 20th century, prior to and during World War II, it spread to North and South America and eventually to Australia in the 1960s. Oil was first extracted in Canada for edible use in 1956. The crude oil is extracted from the seed, degummed, alkali refined, and bleached before it is used in the manufacture of margarine and oil. Over the years the quality of this oil has improved through development of the industry. In 2001, area under production of oilseed rape was 23.9 Mha (FAO 2002) spanning most continents of the world.

Blackleg disease has been documented since the early 19th century (Henderson 1918). Since then blackleg has been reported in most oilseed rape producing regions of the world, due mainly to worldwide seed transmission. Since 1966, devastating epidemics have occurred on oilseed rape throughout many parts of the world, especially Canada and Europe (Gugel and Petrie 1992). The disease is rare in the large area of oilseed rape grown in Asia, however, West *et al.* (2000) reported stem lesion symptoms from three different field sites in central China. Affected tissues, when incubated, formed pseudothecia and yielded ascospores of *L. maculans*.

The oilseed rape industry began in Australia in the late 1960s. The first commercial crop was grown in 1970 (Roy and Reeves 1975). Both *B. napus* (cvs Target and Midas) and *B. rapa* (cvs Arlo and Span) were grown in the early years, *B. napus* in the medium to high rainfall areas and *B. rapa* in the drier, shorter season areas (Colton and Potter 1999). Production increased very quickly in the early 1970s, largely in response to the imposition of wheat

quotas that restricted the area of wheat sown. For example, in Western Australia (WA) the area sown to oilseed rape increased from 120 ha in 1969 to 49,000 ha in 1972 (Plummer *et al.* 1994; Salisbury *et al.* 1995). Blackleg became prominent in Australia in the early 1970s and WA was worst affected. Here, yield losses of up to 80% occurred, which led to a reduction in the area sown to 3,200 ha in 1973 and 2,000 ha in 1974 (Bokor *et al.* 1975). Good management through crop rotations, sowing time and fungicide application helped to control the disease but more effective strategies of blackleg control were needed for the future of the oilseed rape industry in Australia.

2.2.2 Australian cultivars

The first oilseed rape breeding program began in Horsham, Victoria (Vic.), in 1970 and by 1973 both WA and New South Wales (NSW) had established breeding programs. Early breeding involved two species, *B. campestris* and *B. napus*. The first Australian cultivars released were Wesreo (1978) and Wesway (1979), both from WA (Colton and Potter 1999).

The name 'canola' was introduced in Canada in 1979. It refers to *B. napus* cultivars of oilseed rape which have less than 2% erucic acid (or toxic fatty acid) and less than 40 μmoles/g of total glucosinolates (Salisbury *et al.* 1995). Canola is also lower in polyunsaturated fats than other oilseeds (Lamont 1990). Marnoo (Vic), Wesbrook and Wesroona (WA) were the first Australian cultivars to fall in the 'canola' category. Since their release early in the 1980s, breeders have concentrated on increasing resistance to blackleg in canola cultivars. In 1982, Jumbuck was released in NSW, providing increased yield, quality and blackleg resistance.

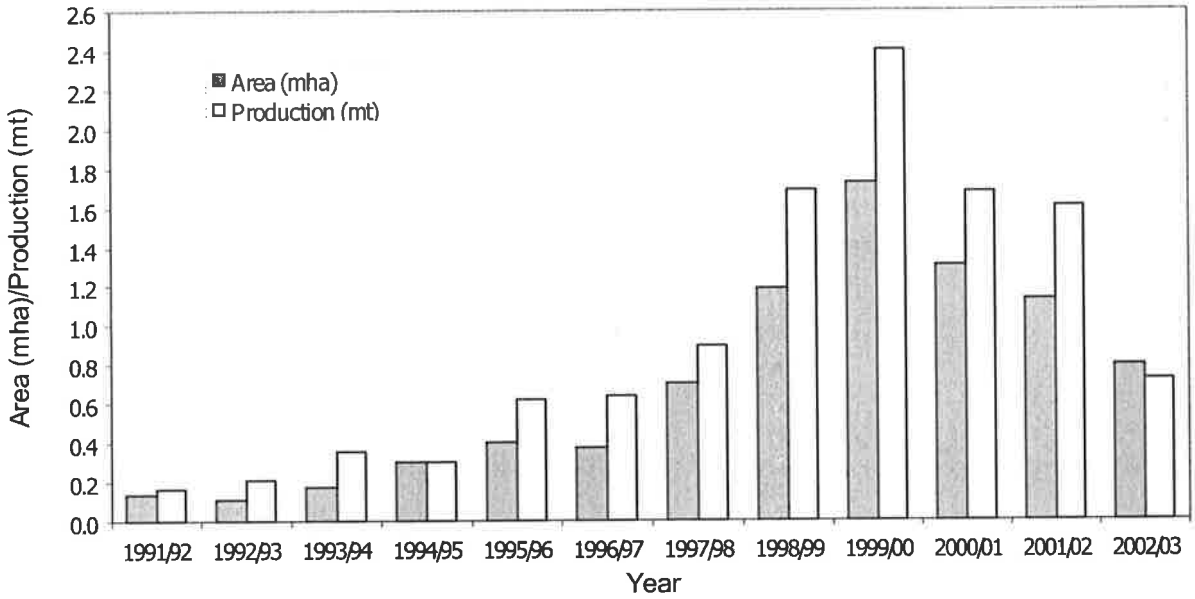
Maluka and Shiralee were released by the NSW breeding program in 1987. These cultivars combined canola quality with blackleg resistance and high yields and resulted in a complete

swing back to *B. napus* cultivars (Colton and Potter 1999). The first *B. napus* hybrid canola, Hyola 30, was released by Pacific Seeds in 1988 and was followed by Hyola 42 in 1991.

The cultivar Siren was the first commercialised triazine herbicide tolerant canola, released in Vic. in 1993. It was late maturing and had low yield and oil content but could be used where cruciferous weeds reduced the success of traditional canola cultivars. New triazine tolerant cultivars soon followed with the early maturity Karoo (Vic) and Drum (NSW) and mid-season maturity Pinnacle (Vic) and Clancy (NSW).

Over the past 10 years there has been a large increase in the area of canola sown for a number of reasons; 1. profitability of the crop, 2. use of canola as a break crop between cereals, 3. use of canola as a replacement for poorly performing legumes and 4. cultivars with improved quality and greater blackleg resistance which better suit the Australian environment. The total area of canola sown in Australia increased ten fold from 175,000 ha in 1993 to 1.7 Mha in 1999, producing an estimated 2.4 Mt of seed (Figure 2.1; Anonymous 1994a; Anonymous 1999). In South Australia, the area has increased twenty fold from 12,000 ha in 1992 to 218,000 ha in 1999, producing an estimated 280,000 t of seed (Lewis 1998; Anonymous 1999). However, in 2001, the area sown to canola has decreased to 972,000 ha in Australia and 145,000 ha in South Australia (Anonymous 2002a) due mainly to decreased market prices and estimated decreases in production in 2002 are due to drought. The dramatic expansion in area sown to canola has increased the risk of disease on the crop by shortening the interval between rotations and, therefore, increasing the inoculum load from crops with blackleg. This highlights the importance of continued breeding for new resistant cultivars. During the past 7 years, over 30 new cultivars have become available to canola growers, widening the range of cultivars on the market with improved blackleg resistance that are suitable for a more diverse selection of environments, including low rainfall areas (Appendix 1).

Figure 2.1: Area sown and production of canola in Australia (compiled from newsletters from the Canola Association of Australia and the Australian Oilseeds Federation, 2002/03 figures are estimates)



2.3 DISEASE SYMPTOMS

2.3.1 Macroscopic description

Leaf lesions are dirty white or pale grey in colour, round to irregularly shaped, and usually dotted with numerous small, black pycnidia (Hammond and Lewis 1987a; Anonymous 1996; Figure 2.2A&B). Often, a conspicuous green margin of chlorophyll can be seen around the lesion. Lesions can be found at the base and crown regions of stems as well as on upper sections, especially at points of leaf attachment. Stem lesions are often oval, long, irregular and grey or cream in colour (Figure 2.2C), and can appear as long black transverse streaks in the cortex. Numerous pycnidia form in the centre of the lesion. These pycnidia produce pycnidiospores in cirrhi which appear as a purple/pink exudate (Figure 2.3D). The lesions may also appear as a general blackening around the base of the stem, again with numerous pycnidia. Eventually the stem or crown become cankered (Figure 2.2D), and the plant may lodge. On pods, lesions are also round to irregular in shape, with pycnidia, and the pod is often constricted (Figure 2.2E).

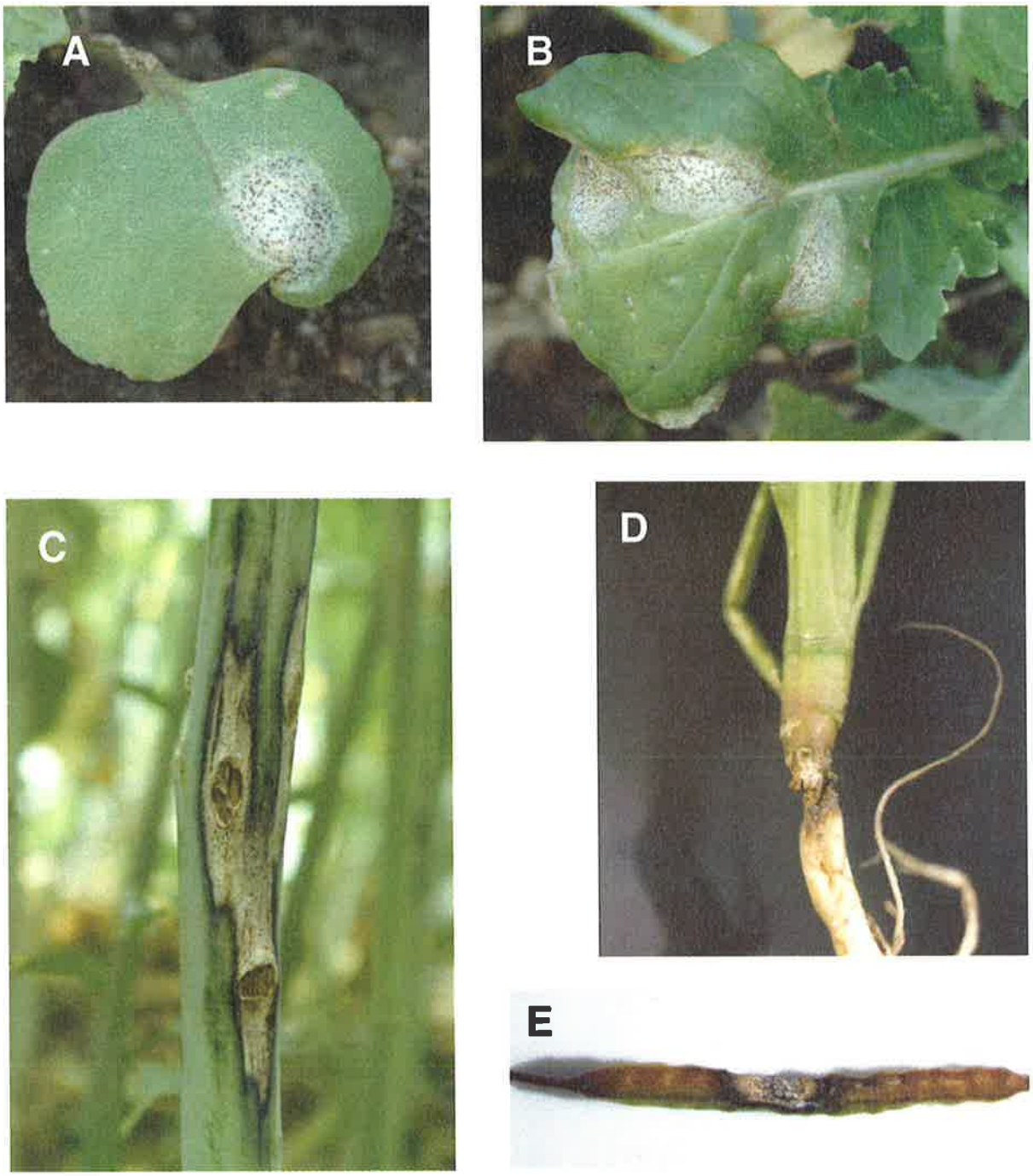


Figure 2.2: Macroscopic symptoms of *L. maculans* infection on canola. A. cotyledon lesion with pycnidia, B. leaf lesions, C. stem lesions, D. severe canker of the crown and E. pod lesion

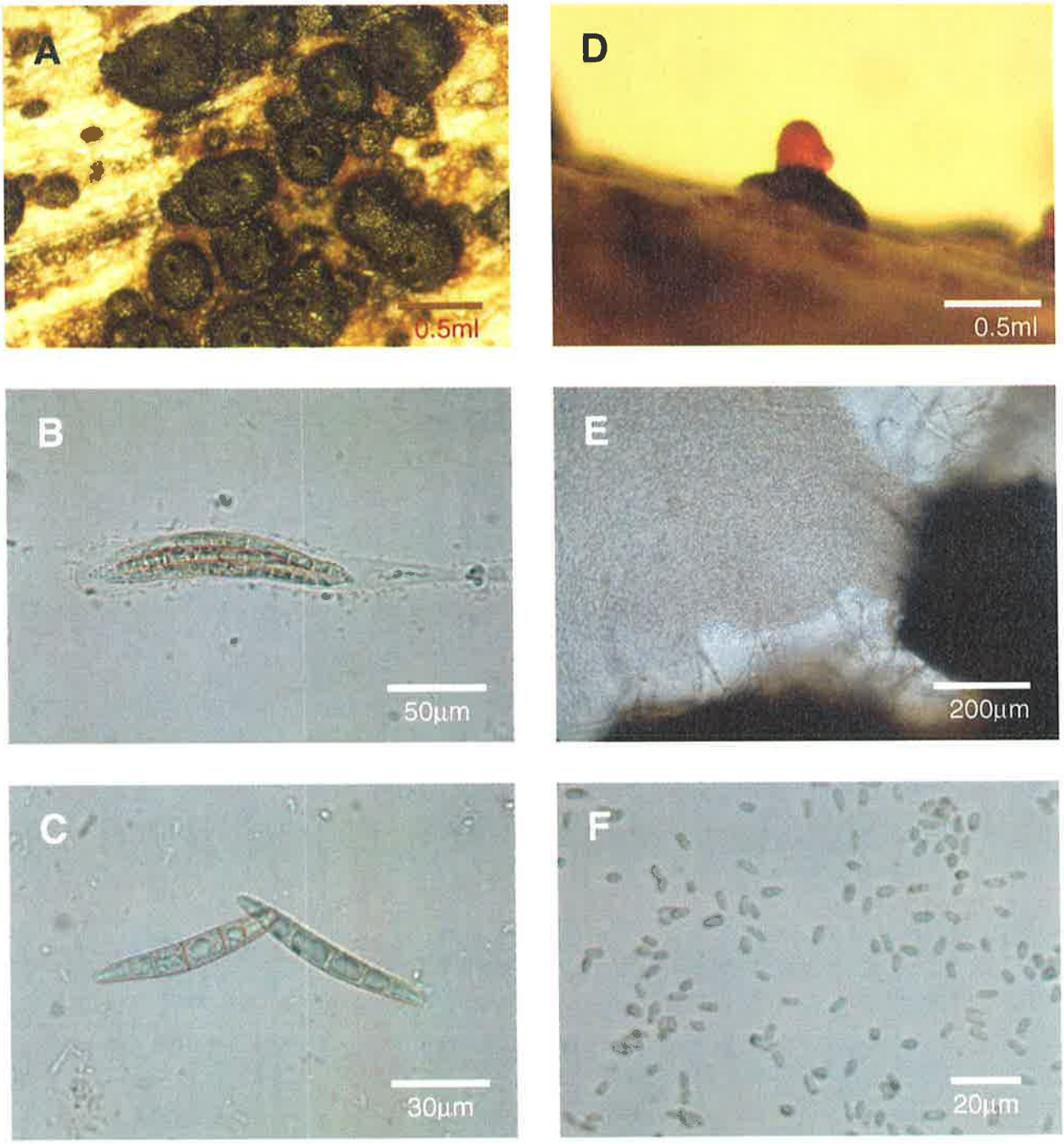


Figure 2.3: Microscopic symptoms and structures of *L. maculans*. A. pseudothecia with ostioles open, B. ascus containing ascospores, C. ascospores, D. pycnidia *in situ* releasing pycnidiospores, E. pycnidia *in vitro* releasing pycnidiospores and F. pycnidiospores

2.3.2 Microscopic description

Pseudothecia (ascomata, perithecia) are found on stems and leaves, and are immersed, globose, black with protruding ostioles, 300-500 μm diameter (Figure 2.3A; Punithalingam & Holliday 1972). Asci are cylindrical to clavate, sessile or short stipitate, 8-spored, 80-125 x 15-22 μm; ascus wall bitunicate (Figure 2.3B). Ascospores are biseriate, cylindrical to ellipsoidal, ends mostly rounded, yellow brown, slightly or not constricted at the central septum, guttulate, 35-70 x 5-8 μm (Figure 2.3C). Pycnidia on stems and leaves are of two types: i) sclerotioid: immersed, variable in shape, convex, with narrow ostioles, 200-500 μm across, ii) globose: black, wall composed of several layers of cells, 200-600 μm diameter (Figure 2.3D). Pycnidiospores (conidia) are hyaline, shortly cylindrical, mostly straight, and some are curved, guttulate, unicellular, 3-5 x 1.5-2 μm (Figure 2.3E&F, Figure 2.4).

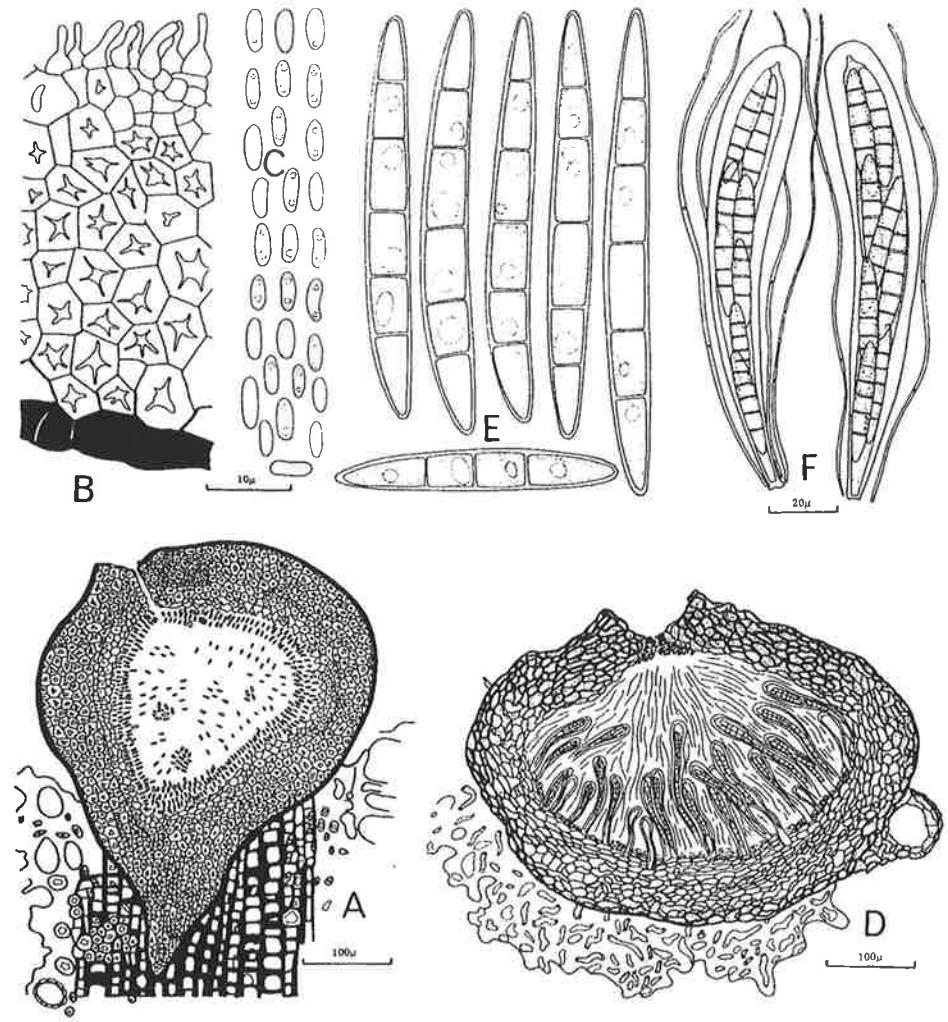
2.4 EPIDEMIOLOGY OF BLACKLEG

2.4.1 Life cycle of *Leptosphaeria maculans*

It is important to understand the life cycle of *L. maculans* in order to minimise blackleg on canola crops. An understanding of the life cycle also allows us to explore plant-pathogen interactions in relation to resistance mechanisms. The life cycle is summarised in Figure 2.5.

L. maculans is heterothallic. Ascospores are the source of primary inoculum (Barbetti 1975; McGee 1977; Williams 1992). They are produced in black fruiting bodies called pseudothecia, which survive on canola stubble (Williams 1992), and can be visible on sites of severe stem canker as early as shortly after harvest (Hammond 1995, cited by West *et al.* 1999). These wind-borne spores are released with the onset of rainfall, or even dew. In canola growing areas of southern Australia, wind-borne spores are most prevalent between May and August when seedlings emerge (McGee 1977). Ascospores adhere to the cotyledons

Figure 2.4: Microscopic morphology of L. maculans (Punithalingam & Holliday 1972)

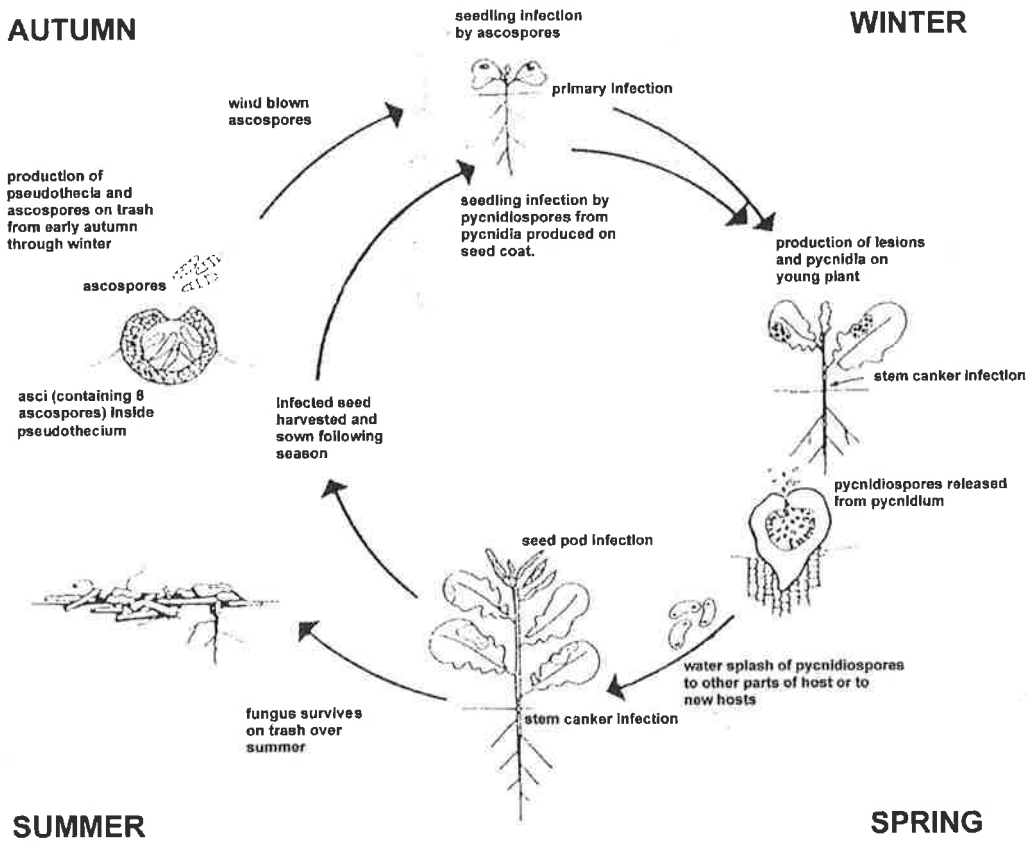


A, Vertical section of pycnidium; B, part of pycnidial wall; C, conidia; D, v.s. of ascocarp; E, ascospores; F, asci and pseudoparaphyses.

and leaves of seedlings, and germinate in humid conditions to produce hyphae that cause infection via stomatal pores and wounds (Chen and Howlett 1996; Sosnowski *et al.* 2001b). Hyphae enter the leaf and colonise intercellular spaces between mesophyll cells. According to Hammond and Lewis (1987a), the fungus initially enters the tissue as a biotroph, as hyphae spread into the leaf tissue to form a hyphal plate, which extends further, causing the first visible signs of infection as the palisade cells begin to collapse 1-2 mm behind the hyphal front. The dead tissue is used to support the production of pycnidia containing pycnidiospores. These spores, which act as secondary inoculum, are spread by rain-splash to other leaves and neighbouring plants. Lesions can also form on the pods, allowing the fungus

to spread to the seed, which can become a source for future infection (Wood and Barbetti 1977a). The hyphae then continue to grow down the petiole in the xylem vessels or between cells of the xylem parenchyma and cortex. The fungus then invades and kills cells of the stem cortex, resulting in a canker that may completely girdle the base of the stem (Salisbury *et al.* 1995). The stem cankering process is necrotrophic. Girdling of the stem can disrupt water transport, causing premature ripening of the pods and yield loss (Davies 1986, cited by West *et al.* 1999) and, in more severe cases, the stems may be weakened considerably, causing lodging and eventual death of plants.

Figure 2.5: Life cycle of *L. maculans* (Bokor *et al.* 1975)



2.4.2 Inoculum

Ascospores are considered the most important form of inoculum and ascospore-derived infection is responsible for most crop loss due to blackleg (Hall 1992). The period of maximum ascospore release often coincides with the period of greatest crop susceptibility (Hall 1992). In Ontario, Canada, ascospores may be released from stubble of recently harvested crops from September to November, to infect seedlings of new winter oilseed rape crops (Rempel and Hall 1993). In western Canada, ascospores are released from stubble from May to August, and infect seedlings of spring oilseed rape crops (McGee and Petrie 1979). In western Europe, ascospores are released from stubble of the previous season's winter oilseed rape crop from late September throughout the autumn/winter period (Gladders and Musa 1980; Thurwachter *et al.* 1999). In eastern Europe, some ascospore release can occur from September to November, but the main period of release is from May till August, after the cold winter (Jedryczka *et al.* 1999b, cited by West *et al.* 2001). In southern Australia, the frequency of ascospore showers is generally greatest between May and August when seedlings emerge (McGee 1977). Ascospores can be carried by the wind for 2-5 km (Hall 1992) and can be transported up to 8 km from their source (McGee 1977). According to Barbetti *et al.* (2000), the greatest risk of infection is within 500 m of the source, with spore numbers decreasing with distance, and the current recommendation in Australia is to sow canola at least 2 km from last year's stubble (Barbetti and Khangura 1999). The fungus does not survive as either ascospores or pycnidiospores alone in the soil for more than a few months (Gabrielson 1983), and long term survival depends on retention of stubble.

L. maculans can persist on canola crop residues for many years as pseudothecia containing ascospores, acting as blackleg inoculum for any canola crops grown in or adjacent to fields previously sown to this crop. In western Canada, ascospores can continue to be produced on stubble in the field for up to 5 years after the crop has been harvested (Petrie 1995b; Baird *et*

al. 1999), due to very cold winters and dry, hot summers slowing decomposition (Petrie 1986). The mild, wet climate in the United Kingdom promotes rapid decomposition of stubble, usually within 2 years (West *et al.* 1999). In south western Australia, stubble remains an inoculum source for up to 4 years, as decomposition is slow in the hot, dry summers. In south eastern Australia, where conditions are milder, up to 90% of stubble can be decomposed within 1 year, and so inoculum is produced mainly from crops grown in the previous year (Bokor *et al.* 1975; McGee 1977; Petrie 1978; Barbetti and Khangura 1997). However, when conditions are drier, it may take longer for stubble to decompose. It is evident that stubble decomposition varies in different growing regions due to climatic factors, and recommendations should, therefore, be made on a regional basis.

Pseudothecial maturation is dependent on both temperature and wetness, with an optimum at 14-15°C (Petrie 1994; Pérès *et al.* 1999). In south western Australia, mature pseudothecia do not usually form until the autumn because maturation stops in the summer (West *et al.* 2001). Pseudothecia have been seen on stubble as early as at harvest time in North America (Hershman and Perkins 1995), but dry weather over the summer period can delay further maturation and spore release. The sub-zero winter temperatures of western Canada can delay maturation for 9-10 months after harvest (Kharbanda 1993, cited by West *et al.* 2001).

The present knowledge on inoculum and its ability to cause epidemics has been gathered using traditional methodology. New deoxyribonucleic acid (DNA)-based technology, which is often much more sensitive than traditional methods, may provide additional information on pathogen survival in soil and on stubble. The importance of soil-borne inoculum of *Mycosphaerella pinodes* and *Phoma medicaginis* var. *pinodella*, causal agents of blackspot in peas, was realised with the use of polymerase chain reaction (PCR)-based methods (Davidson *et al.* 1999). Using a DNA-based assay involving primers designed to amplify the internal

transcribed spacer (ITS) region, they showed that the blackspot fungi can persist for up to 4 years after field peas are grown and it can survive in the absence of pea stubble. This technology has not yet been applied for pathogen survival studies of blackleg on canola.

Secondary infection by pycnidiospores is rare in Canada and Europe but more common in Australia (Wood and Barbetti 1977b; Gladders and Musa 1980; Thurwachter *et al.* 1999). Barbetti (1976) demonstrated the pattern of disease spread in the field in WA following inoculation with pycnidiospores and concluded that pycnidiospores could play a significant role in the establishment of leaf, crown and stem infections. Barbetti (1976) showed that pycnidiospores could spread the disease up to 1 m from the original foci in susceptible crops.

Contaminated seed is not generally considered a major source of inoculum but it can introduce the fungus into an area (Wood and Barbetti 1977a; Gabrielson 1983). Hall *et al.* (1996) estimated that a seed lot containing 1% artificially contaminated seed could give rise to crown cankers in 3.3% of the plants, cause widespread distribution of diseased plants in the field and yield losses of 1-2%. Wood and Barbetti (1977a) showed that plots sown with 5.9% and 0.08% naturally infected seed produced 19.0% and 1.1% of plants with crown cankers, respectively. However, McGee (1977) showed that disease incidence or severity is not related to the proportion of contaminated seed.

Plants other than oilseed rape and their residue can also act as a source of inoculum. Isolation of *L. maculans* has been reported from a wide range of plants, mostly in the Cruciferae, but also in the Compositae, Onagraceae and Gentianaceae (Gabrielson 1983). Barbetti (1978) showed that wild radish residue in young crops of oilseed rape bore pseudothecia with viable ascospores, meaning that this weed could have been a significant source of inoculum.

2.4.3 Infection

The amount of leaf and stem infection is influenced by a number of factors, including temperature, humidity, time of infection and spore concentration. In the field, it is most likely that a combination of these factors will determine progress of infection and the occurrence of a disease epidemic. An epidemic is defined by Agrios (1997) as an increase in disease in a population over time, usually a widespread and severe outbreak of a disease.

2.4.3.1 Humidity, wetness period and temperature

The optimal conditions for germination of ascospores and pycnidiospores differ. Hall (1992) reported that 100% of ascospores germinated *in vitro* within 8 h at 28°C whereas 100% of pycnidiospores germinated within 24 h at 24°C. Ascospores require no more than 4 h of continuous wetness for germination to occur and pycnidiospores require at least 16 h. Since ascospores are released in autumn/winter when temperatures rarely reach 28°C, 100% germination *in vivo* is unlikely to be achieved. *In vitro* studies by Huang *et al.* (2001) showed that as temperature increased from 5-20°C the percentage of germinated ascospores also increased.

Infection is greatest in humid conditions and less disease has been recorded in drier seasons (McGee 1977; Xi *et al.* 1991). When plants were inoculated with a pycnidiospore suspension and subjected to 100% relative humidity for durations ranging from 0 to 120 h, the incidence of diseased plants rose from 36% to 98% respectively (Wood and Barbetti 1977b). On leaves inoculated with ascospores, localised lesions occurred at 4°C and systemic infections occurred at 15°C (Hammond and Lewis 1987a). In controlled environment experiments conducted by Biddulph *et al.* (1999), ascospores of *L. maculans* infected oilseed rape leaves and caused lesions (phoma leaf spot) at temperatures from 8°C to 24°C and leaf wetness durations from 8 h to 72 h. The conditions that produced the most leaf lesions were a leaf wetness duration of

48 h at 20°C, and numbers of lesions decreased with decreasing wetness duration and increasing or decreasing temperature. Furthermore, leaf lesions first occurred after 5 days at 20°C but after 2 weeks at 8°C. Toscano-Underwood *et al.* (2001) carried out similar experiments, and reported that the greatest numbers of leaf lesions from ascospore infection occurred with a leaf wetness duration of 48 h and at temperatures of 15-20°C. The latent period between infection and formation of leaf lesions may differ between cultivars and between leaves in different positions (Poisson and Pérès 1999). Biddulph *et al.* (1999) provided evidence that controlled environment data can be applied to disease development in the field. However, experiments by Biddulph *et al.* (1999) suggested that it would not be possible to use the occurrence of optimal infection conditions alone in a weather-based system for predicting the occurrence of leaf lesions, as epidemics did not always eventuate when conditions were fulfilled.

Hammond and Lewis (1986a) reported that the time between the appearance of leaf lesions and stem lesions on *B. napus* cultivars was 75 days at a mean temperature of 15°C and 175 days at 3°C. Cankers develop most rapidly and are most severe when temperatures are between 20-24°C, they develop slowly at 4-8°C and do not develop over 28°C (Barbetti 1975; McGee and Petrie 1979; Gladders and Musa 1980). Furthermore, temperature can influence the type of canker that develops. Barbetti (1975) discovered that small, soft cankers with few pycnidia were produced at day/night temperatures of 12/7°C, whereas large, dry cankers with many pycnidia appeared at 18/11°C and 24/15°C. Nathaniels and Taylor (1983) showed that early infection by *L. maculans* remained latent during winter temperatures (i.e. 14°C), whereas symptoms developed rapidly when temperatures increased to 18-20°C.

It is evident that the optimal climatic conditions for infection can vary depending on spore type and cultivar. A lot of information exists on ascospore infection around the world,

however, pycnidiospore infection plays a more significant role in epidemics in Australia than in North America and Europe (section 2.4.2). Therefore, by examining the conditions which promote infection by pycnidiospores on current Australian cultivars, it may be possible to improve management of canola crops to minimise blackleg.

2.4.3.2 Timing of infection

The timing of ascospore infection, in relation to the growth stage of the plant, may influence the success of the infection (West *et al.* 2001). Hammond and Lewis (1986a) observed that leaf lesions occurred mostly on leaves three to ten, and subsequently reported that as the leaves aged, a progressively larger number of infections failed to become systemic (Hammond and Lewis 1987a). Similarly, McGee and Petrie (1979) found that the first six leaves appeared to be more susceptible to infection than later leaves. Symptoms appeared more rapidly on leaf six than on leaf four or leaf two, according to observations by Poisson and Pérès (1999), however, isolations revealed that the fungus was present in leaf two, four and six, 2, 6 and 14 days after inoculation, respectively, even though lesions had not formed, indicating that earlier leaves are more susceptible to infection. Xi *et al.* (1991) showed that the incidence of petiole and stem infections was higher in younger plants due to the effects of stem age, leaf number and leaf age on susceptibility to infection in Canada. In contrast, Badawy *et al.* (1991) showed that symptom expression, even by non-aggressive isolates, was more severe on ageing host tissue than on younger tissue in Germany. The apparent contradiction may be due to the fact that one study focussed on incidence, and the other on severity. Stems are most susceptible at the cotyledon and one to two leaf stage (McGee and Emmett 1977; McGee and Petrie 1979). As stem extension occurs, pathogen spread from lesions on new leaves can produce upper stem lesions as opposed to basal stem cankers, which do not affect yield as severely as cankers (West *et al.* 1999). The potential of the pathogen to reach the stem depends on whether or not the ambient temperature promotes

accelerated leaf abscission relative to the progress of the fungus (Hammond and Lewis 1986a). It also depends on the size and age of the leaf when infection occurs and the proximity of the lesion to the petiole. Based on the studies discussed above, it seems most likely that epidemics will occur when inoculum levels are high at early stages of plant development.

2.4.3.3 Inoculum concentration

The number of ascospores discharged from plant residue directly affects the severity of the disease in the crop and surrounding crops (McGee 1977) and Hammond and Lewis (1987a) showed that an increased concentration of pycnidiospore inoculum could increase the incidence and severity of disease. In contrast, Wood and Barbetti (1977b) found that increasing inoculum concentrations of ascospores and pycnidiospores from 150 and 500 spores/ml to 10^4 and 10^7 spores/ml respectively, had little effect on disease incidence. This contradiction may be due to the use of different inoculation methods. Wood and Barbetti (1977b) placed 10 µl drops of inoculum on the intact cotyledons whereas Hammond and Lewis (1987a) placed 8 µl drops of inoculum on wounded sites on leaves of varying ages. The wounding immediately removes a barrier in the infection process, making comparison between these findings difficult.

Ascospore inoculum has been shown to be more efficient at inducing disease than pycnidiospore inoculum at the same concentration (Wood and Barbetti 1977b). However, pycnidiospores are easy to produce *in vitro* on agar media, whereas ascospores can only be produced from pseudothecia on canola stubble.

2.4.3.4 Physical damage

Physical damage such as sand blasting, hail damage and insect damage will wound the epidermis of the leaf, facilitating penetration by hyphae and colonisation of the leaf tissue (Barbetti and Khangura 1999; West *et al.* 2001).

2.4.4 Disease modelling and forecasting

Accurate forecasting of severe stem canker epidemics can improve disease control, according to West *et al.* (1999). This is especially important in the United Kingdom, where foliar fungicides can be applied throughout the season. West *et al.* (1999) stated that early warnings of risks of severe stem canker epidemics could be provided at the beginning of the season through regional forecasts based on disease survey and weather data. The accuracy of such forecasts could be improved by including factors relating to the maturation of ascospores in pseudothecia, the release of ascospores and the occurrence of infection conditions, as they affect the onset, intensity and duration of the leaf lesion phase.

Thurwachter *et al.* (1999) evaluated ascospore discharge, ratio of aggressive to non-aggressive isolates, and leaf and stem infections for the forecast of stem canker. They found no apparent relationship between ascospore discharge and either stem or leaf infection, but there was a positive relationship between leaf infection and stem canker. They also discovered that there was no relationship between the proportion of aggressive isolates and blackleg intensity. It was concluded that these three criteria alone are insufficient for the development of a system of blackleg forecasting and further factors such as climate, seed and soil-borne inoculum, and cultural practices should be included in models for forecasting the impact of blackleg on oilseed rape. Models were constructed by Sun *et al.* (2000) to describe the relationship between the incidence of phoma leaf spot and stem canker. Using these models, Sun *et al.* (2001) subsequently reported that the severity of crown canker and phoma

stem lesions increased linearly with accumulated degree-days and that the severity of crown canker in spring was related to crown canker severity at harvest.

The relationships between yield loss and stem canker in oilseed rape were analysed using data from field experiments by Zhou *et al.* (1999). They found that "critical point" and "area under the disease curve" models were better than multiple point models for their experiments. The greatest yield losses were generally associated with the largest severity scores, and were also associated with the early development of stem lesions. Furthermore, they showed that percentage yield loss was related to incidence or severity of both basal stem cankers and upper stem lesions in their experiments. A later study revealed that flowering and pod development were critical points for relating percentage yield loss to stem canker (Zhou *et al.* 2000). Xi *et al.* (1990) examined the relationship between disease incidence and severity and showed that there was a general similarity between the two measures of blackleg. Khangura and Barbetti (2001) reported that high levels of blackleg found in commercial crops are indicative of significant losses in seed yields. Yield losses at harvest are usually up to 10%, although they can reach as much as 30-50% (Hall *et al.* 1993; Barbetti and Khangura 1999; Zhou *et al.* 1999).

The ability to forecast the timing and intensity of epidemics is important for management strategies to minimise yield loss. In Australia, there is no such system available, so there is a need for epidemiological information to be collected in order to develop models which examine the effect of a range of factors on blackleg incidence and severity.

2.5 MOLECULAR DIAGNOSTICS

Traditional biological methods of disease diagnosis are essential in current plant pathology, but they may be expensive, time consuming and labour intensive (Randles *et al.* 1996). They

include microscopic examination, culturing of the pathogen on artificial media and plant assays, the use of Koch's postulates, which requires purification of the pathogen and then expression of the disease on test plants. Diagnostic tests, based on a number of molecular technologies, are now available to detect plant pathogens; some replace existing systems, though many complement traditional methods (Fox 1997). They can be rapid, inexpensive, highly sensitive and specific, providing quick and accurate diagnosis so that control measures can be used more effectively. Serological techniques were the first molecular diagnostic tools, and have been available since the 1960s. Nucleic acid techniques have been available more recently.

2.5.1 Serology

Immunoassays

Enzyme-linked immunosorbent assay (ELISA), which has been used predominantly in the detection of viruses, has also been developed for a wide range of plant pathogens (Miller and Martin 1988). It works by amplification of antibody-bound antigen through the binding of an enzyme (such as alkaline phosphatase) to the antibody and then adding a chromogenic substrate which reacts with the enzyme to produce a colour (Randles *et al.* 1996). Unlike traditional methods, ELISA does not require culturing of the pathogen, it produces results within 24 h, has high specificity and sensitivity, can quantify the pathogen and the technique can be automated (Putnam 1995). Examples of the use of this technology include the detection of prunus necrotic ringspot virus in roses (Moury *et al.* 2000), barley yellow dwarf virus (D'Arcy *et al.* 1992) and the fungal pathogen *Phomopsis longicolla* in soybeans (Brill *et al.* 1994). However, fungal pathogens are complex and antisera may lack specificity (Narayanasamy 1997). To date, ELISA has not been used in the detection of *L. maculans* in canola.

2.5.2 Nucleic acid techniques

DNA stores and transmits genetic information and contains specific sequences unique to each organism (Finch 1994). All viable propagules contain the entire nucleic acid complement of the organism and this is not altered by life cycle stages, response to the environment or by the host. Antigens may only be present at certain times in an organism's life cycle whereas nucleic acid detection considers part or the whole of the genome rather than a gene product, as with serology (Randles *et al.* 1996).

Probes

Nucleic acid probes are sequences of nucleic acids (DNA or RNA) that are labelled with a marker and used to detect complementary nucleic acid sequences (Miller 1995). A probe is employed by first fixing partially purified nucleic acids from the affected plant to a substrate such as nitrocellulose or nylon membrane (Putnam 1995). The target nucleic acids are denatured to a single strand and 'probed' with labelled cDNA or cRNA. In suitable conditions of buffer and temperature, the probe hybridises with the target DNA or RNA and the hybrids of double-stranded nucleic acid are detected by visualisation of the label. DNA probes have been used for the diagnosis of many fungi in plants and soil, such as; *Phytophthora cinnamomi* in avocado roots (Judelson and Messenger-Routh 1996), a range of *Pythium* species (Klassen *et al.* 1996), *Gaeumannomyces graminis* var. *tritici* (*Ggt*) in soil (Herdina and Roget 2000) and *Uncinula necator* in grapes (Evans *et al.* 1996).

Polymerase chain reaction

PCR can be used to detect minute quantities of plant pathogen DNA in soil or plant material (Putnam 1995). It works by exponentially amplifying the target DNA *in vitro* with repeated cycles of synthesis. The reaction mixture consists of the target DNA, buffer, free nucleotides, a DNA polymerase and primers (short single strands of DNA which define the sequence to be

amplified). Double strands of target DNA are separated and then hybridised with primers, adding complementary nucleotides according to the sequence present on the target DNA using DNA polymerase. Examples in which PCR was used to detect fungi include; *Ggt* in soil (Ophel-Keller *et al.* 1995), *Glomus mosseae* in leek roots (Edwards *et al.* 1997) and *L. maculans* in canola leaves (Mahuku *et al.* 1996) or from ascospores collected in a spore trap (Calderon *et al.* 2002).

Restriction fragment length polymorphism analysis

The term restriction fragment length polymorphism (RFLP) refers to the variation in the size of products obtained when total DNA from various, closely related sources is cut with sequence-specific restriction endonucleases (Michelmore and Hulbert 1987). In short, pathogen isolates to be compared are cultured, DNA isolated, and then various restriction enzymes are used to cleave the DNA, and the products fractionated by size using agarose gel electrophoresis and visualised as bands. It may be useful for distinguishing races, pathotypes, strains or anastomosis groups (AG) which belong to the same species (Randles *et al.* 1996). The banding patterns can be considered to act as a DNA profile (fingerprint) of a given isolate and specific patterns may be characteristic of a given isolate. This technology has been utilised for the identification of AG-3 of *Rhizoctonia solani* (Balali *et al.* 1996), as well as to distinguish sub-groups of *L. maculans* in canola (Koch *et al.* 1991).

Random amplified polymorphic DNA

Random amplified polymorphic DNA (RAPD) can be used to compare DNA from different individuals by observing variation in the banding patterns of randomly generated DNA fragments, obtained following amplification by PCR using arbitrary primers and combining the principles of PCR and RFLP (Randles *et al.* 1996). RAPD has been used for pathogens such as *Ceratocystis fimbriata* var. *plantani* (Santini and Capretti 2000) and *Aschochyta*

rabiei (Khan *et al.* 1999) as well as for *L. maculans* (Goodwin and Annis 1991; Mahuku *et al.* 1997; Voigt *et al.* 1998). However, a disadvantage of RAPD is the lack of reproducibility.

Amplified fragment length polymorphism analysis

Amplified fragment length polymorphism (AFLP) analysis allows high stringency PCR amplification of target DNA fragments randomly chosen from restriction fragments. Though similar to the RAPD technique, as it analyses the whole target genome, it has the advantage of giving results which are very reproducible since it uses stringent PCR conditions (Edel 1998). Purwantara *et al.* (2000) were able to classify *L. maculans* isolates into five distinct groups using AFLP.

Internal transcribed spacer regions of ribosomal DNA

PCR techniques have included the use of primers designed to amplify the internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA). These regions are poorly conserved and often contain large sequence differences between organisms or even species of pathogens. This enables the development of rDNA specific probes, particularly when species specific identification is required (Annamali *et al.* 1995). Sreenivasaprasad *et al.* (1996) used primers from the ITS region of the rDNA repeat unit in PCR to distinguish between *Colletotrichum* spp. There have been several analyses of the ITS region of rDNA of *L. maculans* to distinguish between A and B-group isolates (Goodwin and Annis 1991; Xue *et al.* 1992, Sosnowski *et al.* 2001b) and to distinguish between subgroups (Morales *et al.* 1993; Balesdent *et al.* 1998).

It is evident that molecular techniques have been succesful as diagnostic tools for certain fungal pathogens, including *L. maculans*, and have the scope to be developed further to enable quantification of the fungus in soil and plant material.

2.6 PATHOGENIC VARIABILITY

Strains of *L. maculans* are classified into two categories; A-group (virulent, Tox+, aggressive) and B-group (avirulent, Tox-, non-aggressive) (West *et al.* 2001). Based on their studies on toxins and metabolites, Pedras and Biesenthal (2000) suggested that tox^{+} and tox^{0} should be more accurately termed $Siro^{+}$ and $Siro^{-}$ respectively, since both strains produce phytotoxins, but sirodesmin is produced only by the A-group strain. Holliday (1989) defined virulence as the observable effects of a pathogen on plants, and it replaces the term "aggressiveness" which has been used extensively in the past but has now been rejected by the Federation of British Plant Pathologists. The A-group, virulent strain is highly pathogenic, as defined by Holliday (1989), in that it has the characteristics to cause severe disease.

It has been suggested that the two strains of *L. maculans* are distinct species because of large differences in chromosome complement, other biochemical, morphological and genetic characteristics and sexual incompatibility (Taylor *et al.* 1991; Williams 1992; Williams and Fitt 1999). Shoemaker and Brun (2001) renamed the B-group as *L. biglobosa* due a prominent beak, located on the ascoma, that is greatly enlarged at the apex.

2.6.1 Techniques for distinguishing strains

As mentioned in section 2.5.2, the A and B-group strains can be distinguished by several molecular techniques such as electrophoretic karyotyping, Southern analysis (or RFLP) of the ribosomal RNA gene repeat, RAPD marker analysis and sequencing of the ITS region of rDNA (Goodwin and Annis 1991; Morales *et al.* 1993; Plummer *et al.* 1994; Salisbury *et al.* 1995; Williams and Fitt 1999). A number of morphological characteristics in culture also differentiate strains, such as rate of hyphal growth, pigment production, germ tube length and pH of spent culture media (McGee and Petrie 1978; De March *et al.* 1986; Koch *et al.* 1989;

Williams 1992; Johnson and Lewis 1994; Williams and Fitt 1999; Sosnowski *et al.* 2001b). Balesdent *et al.* (1992) reported that A-group isolates could be classified by their ability to synthesise a range of phytotoxins *in vitro*, such as sirodesmin PL and phomenoic acid, hence the name tox^+ or $Siro^+$ (Pedras and Biesenthal 2000).

2.6.2 Pathogenicity of strains

Due to the variability of the blackleg fungus, the epidemiology of the disease will depend to some extent on the strain of the pathogen (Hall 1992). Both A-group and B-group strains are able to cause leaf lesions on canola, however the more virulent strains have a greater ability to cause stem cankers (Salisbury *et al.* 1995, Sosnowski 1999).

Leaf lesions formed following infection by B-group ascospores *in vitro* and *in vivo* had few pycnidia and were darker, smaller and less noticeable than the larger, pale grey lesions with many pycnidia produced by infections resulting from A-group ascospores (Toscano-Underwood *et al.* 2001). There was no evidence that the number of leaf lesions produced in relation to number of ascospores inoculated differed between the A-group and B-group strains (Hammond and Lewis 1987b). However, lesions started to appear within 3 days of inoculation for the B-group strain compared to 5 days for the A-group. Toscano-Underwood *et al.* (2001) also observed that the latent period for lesion formation was shorter for B-group isolates than A-group isolates. In addition, Huang *et al.* (2001) found that the time between inoculation and ascospore germination was shorter for B-group isolates than for A-group isolates and that germ tubes from B-group ascospores were longer than from A-group ascospores. However, Huang *et al.* (2001) also reported that i) the mean diameter of germ tubes, ii) the mean number of germ tubes produced from each ascospore and iii) hyphal branching were all greater for A-group ascospores compared to B-group ascospores. Sosnowski *et al.* (2001b) reported that 6 days after inoculation of a susceptible cultivar with

pycnidiospores, a higher percentage of hyphae of a B-group isolate had penetrated the leaf through stomata than did those of an A-group isolate.

The two strains differed markedly in their patterns of stem infection (Hammond and Lewis 1986b). On the stems of susceptible cultivars, the A-group isolates caused extensive lesions in the cortex, which were light brown in colour, often with a distinct dark margin. In contrast, the B-group isolates often did not reach the stem and rarely caused cortical lesions, but penetrated the leaf gap to enter the stem pith without any external symptoms. Johnson and Lewis (1994) also observed that B-group isolates could induce pith lesions on *B. napus* cultivars. This implies that a mechanism is acting in the stems of resistant cultivars, to prevent colonisation of the cortex regions.

Since this fungus reproduces sexually, it is expected that strains will exhibit a range of virulence. The type of strains which exist in an area will influence the possibility and severity of epidemics, making this information important to breeders and growers alike.

2.6.3 Sub-groups

A-group isolates have been separated into pathogenicity groups (PG2, PG3 and PG4) based on severity of symptoms on inoculated cotyledons of three cvs; Westar, Glacier and Quinta (Rimmer and van den Berg 1992; Howlett *et al.* 2001). B-group isolates (PG1) have been classified into three genetically distinct sub-groups (NA1, NA2 and NA3) on the basis of RFLP studies (Koch *et al.* 1991), and this has been supported by a number of different analyses (Howlett *et al.* 2001).

2.6.4 Sexual reproduction

Matings *in vitro* between A-group Canadian strains have produced viable ascospores (Petrie and Lewis 1985). However, attempts to mate A-group with B-group strains have failed (Gabrielson 1983; Salisbury *et al.* 1995; Somda *et al.* 1997) and no evidence exists of inter-strain mating in nature. Somda *et al.* (1997) reported the first successful sexual reproduction between B-group isolates.

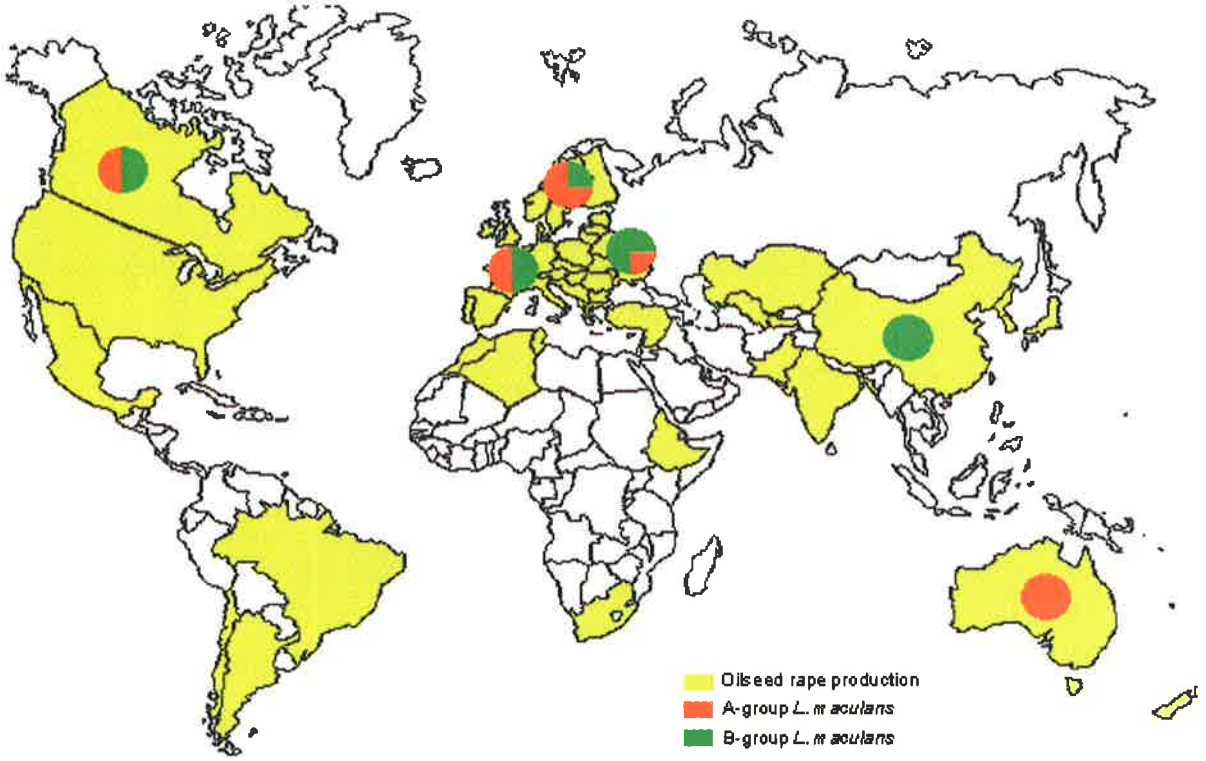
2.6.5 Population structure in different regions

In Australia, both strains have been reported. A-group pathotypes predominate in all canola growing regions of Australia, as shown by Cargeeg and Thurling (1980), Mengistu *et al.* (1991), Ballinger and Salisbury (1996) and Sosnowski *et al.* (2001b), with PG2, PG3 and PG4 all being present. Plummer *et al.* (1994) first reported the existence of B-group isolates in Australia, on the basis that they were unable to infect cotyledons or stems of *B. napus*. Sosnowski *et al.* (2001b) showed that A-group isolates predominated in South Australia, and only two isolates of 40 tested had very high similarities to published gene sequences of B-group isolates (Morales *et al.* 1993). These two isolates were later confirmed as NA2 (B. Howlett, personal communication 2002) using a PCR genomic fingerprinting method based on that of Jedryczka *et al.* (1999). Furthermore, the pathogenicity of A-group isolates varied considerably on a range of canola cultivars. A recent study by Barrins *et al.* (2002) revealed that there was as much genetic diversity between the A-group isolates collected within a single field in Victoria than there was between the isolates which came from fields that were hundreds of kilometres apart.

Evidence from around the world (Newman 1984; Hammond and Lewis 1987b; Gugel *et al.* 1990; Rimmer and van den Berg 1992) confirms the existence of different strains and it seems there is a great deal of variability in different countries. In Canada, B-group strains

predominate, although recently the incidence of A-group strains has increased (Gugel and Petrie 1992), with mainly PG3 and PG4 present in Ontario along with another group, tentatively named PG5 (Mahuku *et al.* 1997), whereas only PG2 was found in western Canada (Mengistu *et al.* 1991). Kutcher *et al.* (1993) found that seven isolates from Western Australia were more pathogenic than 17 isolates from Saskatchewan and four isolates from Manitoba, Canada. Newman (1984) tested 10 isolates from around England and found that only three of them were A-group. More recently, A-group isolates have become predominant in western Europe, where epidemics are usually more severe than in eastern Europe, where the B-group predominates (Howlett *et al.* 2001). In one study in France, more than 90% of A-group isolates were PG3, and the remainder were mostly PG4 (Ansan-Melayah *et al.* 1997). In another French study, all 36 isolates from *B. juncea* cv. Picra were characterised as B-group (Somda *et al.* 1996). West *et al.* (2002) revealed that A-group and B-group isolates were found in different proportions on different parts of the plant and at different times of the year in England and France. Of 120 isolates from Sweden analysed using PCR, 100 were classified as A-group and 20 as B-group (Kuusk *et al.* 2002). Of the A-group isolates, 37 were PG3 and 63 were PG4, based on a cotyledon assay. The trend that A-group isolates are becoming predominant in Europe and the United Kingdom may explain the increase in blackleg stem canker incidence in these areas. Purwantara *et al.* (2000) studied 100 isolates from Australia, Europe and North America and reported that the majority of Australian isolates were A-group, the majority of eastern European isolates were B-group, and western European and North American isolates were evenly divided between the two strains. Furthermore, the Australian and European A-group isolates were more genetically diverse than North American A-group isolates. A survey in central China revealed that stem canker occurred rarely, and only B-group isolates were found (West *et al.* 2000). Figure 2.6 shows the distibution of *L. maculans* in some oilseed rape growing areas of the world, and highlights

Figure 2.6: Countries contributing to world oilseed rape production in 2001 (FAO 2002) and distribution of *Leptosphaeria maculans* A and B-group strains since 2000 in countries where studies have been carried out (compiled from data by Kuusk *et al.* 2002; Purwantara *et al.* 2002; West *et al.* 2002). Oilseed rape was also produced in south western Russia in 2001.



the need for further studies to determine the population structure in the United States of America and in countries of South and Central America, Africa and Asia.

The fact that isolates from Australia are more pathogenic than isolates in other countries contributes to the greater severity of blackleg in this country than elsewhere (Gugel and Petrie 1992). The reason for this difference is not yet fully understood, but it may be due to environmental factors, with conditions in southern Australia particularly suitable for growth of the A-group strain. The predominance of A-group strains in Australia means that the pathogen has a considerable ability to mate and result in outbreeding, possibly leading to strains which can overcome resistance in canola cultivars.

2.7 BLACKLEG DISEASE RESISTANCE

2.7.1 Mechanisms of resistance

Resistance induced by infection may occur by the direct response of either physical features or production of chemicals. The formation of periderm, callose deposition, gel and tylose production have all been linked with resistance of a range of plant species to fungal infections (Misaghi 1982). Modification of the cell wall is common in plants infected by fungi (Politis and Goodman 1978). This can occur as papillae, which are wall appositions formed directly beneath fungal penetration pegs, or lignification. Lignification forms a physical barrier between the cell wall substrates and the enzymes secreted by the pathogen (Friend 1976). Chemicals which plants produce in response to pathogens include glucosinolates, peroxidases, phenolic compounds, catalase, glycosides, ethylene, histones and phytoalexins. It is unlikely that resistance of canola to *L. maculans* is due to one of these defence mechanisms only. As Misaghi (1982) emphasised, plant defence is generally the function of a number of mechanisms operating in an integrated, coordinated manner.

2.7.1.1 Glucosinolate content

Glucosinolates (sulphur-containing glucosides), a group which comprises approximately 100 secondary plant metabolites, have been examined for their role in resistance against pathogen invasion in *Brassica* species. They are hydrolysed by the plant enzyme, myrosinase, to release compounds such as isothiocyanates, thiocyanates and nitriles, which are toxic to fungi (Manici *et al.* 1997; Sexton *et al.* 1999). Myrosinase is stored in specific myrosin cells, keeping it separate from glucosinolates, the exact cellular distribution of which is unknown. When tissue damage occurs, the two interact and hydrolysis reactions take place. The *in vitro* experiments of Mithen *et al.* (1986) showed that, in the presence of myrosinase, the compounds 3-butenyl-, 3-pentenyl- and 1-methoxy-3-indolylmethyl-glucosinolate exhibited anti-fungal activity, however 2-hydroxy-3-butenyl-glucosinolate did not inhibit growth of *L.*

maculans. Sexton *et al.* (1999) supported this finding by showing that 3-butenyl-glucosinolate had no inhibitory effects on *L. maculans*. Mithen *et al.* (1987) subsequently proposed that glucosinolate levels in *B. napus* leaves were inversely correlated with lesion extension after inoculation with isolates of *L. maculans*. This was later refuted, as the results of Giamoustaris and Mithen (1997) suggested that increasing the total levels of glucosinolates is not likely to lead to an enhancement of resistance to disease. In *Brassica* lines where 2-propenyl-glucosinolate content was very low, infection was not successful, suggesting that sensitivity to glucosinolate hydrolysis products is not the only factor preventing colonisation of this host. Fieldsend and Milford (1994) reported that *B. napus* cultivars with lower concentrations of 3-pentenyl-glucosinolate in their leaves and lower concentrations of 3-pentenyl- and 3-phenylethyl-glucosinolates in their stems, were not consistently infected by pathogens. Sexton *et al.* (1999) found that although the volatile 2-propenyl-glucosinolate, which was released from glucosinolates in tissues of *B. juncea*, is toxic to the blackleg fungus *in vitro*, there was no relationship between blackleg resistance (in stems or cotyledons) and the level of this compound found in *B. juncea*. They suggested that either 2-propenyl-glucosinolate does not play a major role in blackleg resistance, or that isolates able to attack *B. juncea* can detoxify or evade the toxic effects of its hydrolysis products. It seems that although some glucosinolates have the ability to inhibit the growth of *L. maculans*, they are not responsible for the resistance that exists in current cultivars of oilseed rape in Australia. It is most likely that they are a component of a combination of different mechanisms which act to prevent the colonisation of this host by *L. maculans*, as discussed earlier.

2.7.1.2 Leaf resistance

Other investigations have targeted the cotyledon and leaf stage of pathogen colonisation. Biochemical analysis revealed that the hypersensitive response of *B. juncea* to *L. maculans* was correlated with the production of the phytolalexin, brassilexin, which accumulated in *B.*

juncea earlier and more intensely than in *B. napus* following an abiotic elicitation (Rouxel *et al.* 1989). In *B. napus-B. juncea* interspecific progenies, phytoalexin accumulation was less intense than in *B. juncea*, but greater than in *B. napus* cultivars susceptible to *L. maculans* (Rouxel *et al.* 1990). Using light microscopy, Xi and Morall (1993) observed that heavily stained areas (presumably the product of host-pathogen interaction) were associated with the disintegration of hyphae in cells of resistant cultivars. They also observed that the hyphae growing in the susceptible cv. Westar, were thicker (5.7 µm diameter) and more abundant at later growth stages than hyphae growing in the resistant cv. Crésor (hyphae 2.1 µm in diameter). Using electron microscopy, Chen and Howlett (1996) found that in both *B. napus* and *B. juncea*, resistance occurred at the stomatal pore by necrosis of the guard cells, which caused the arrest of hyphal growth. These authors also noted that, in *B. juncea*, resistance occurred in the mesophyll layer of the leaves in the form of deformation of hyphae, accumulation of unspecified dark material and of callose. In cotyledons and leaves of the blackleg resistant wild crucifer species *Arabidopsis thaliana*, *Diplotaxis muralis*, *D. tenuifolia* and *Sisymbrium loeselii* inoculated with an aggressive isolate of *L. maculans*, a hypersensitive reaction comprising rapid cell death, tissue browning and lignification occurred (Chen and Seguin-Swartz 1999). However, isolation of viable fungus from cotyledon and leaf tissue of the wild crucifers indicated that the defence response did not kill the fungus. Sosnowski *et al.* (2001b) found a lower stomatal density in the resistant cv. Dunkeld than in the susceptible cv. Hyola 42. As hyphal penetration was random, the susceptible cultivar was colonised by more hyphae and consequently suffered more severe symptoms. Cytological investigation, by Roussel *et al.* (1999a), of the responses induced in cotyledons of oilseed rape plants showed a correlation between restriction of the pathogen and reinforcement of cell wall barriers, including wall apposition, papillae and vessel plugging.

Studies have also been carried out on the leaves of other hosts to identify mechanisms of resistance. Histochemical studies revealed that resistant wheat leaves infected with the rust fungus *Puccinia recondita* f. sp. *tritici*, had lignification of cell walls of the stomata, mesophyll and vascular tissue (Southerton and Deverall 1990). Dushnicky *et al.* (1998) illustrated the resistance of wheat leaves to *Pyrenophora tritici-repentis* by lignification of the intercellular spaces of the mesophyll around sites of infection using histochemical techniques as well as scanning electron microscopy. Flavonoids, peroxidase, catechins and lignins were found in resistant grapevine leaves inoculated with *Plasmopara viticola* by Dai *et al.* (1995). Faulkner and Kimmins (1975) reported the presence of lignin, suberin and callose in the border of lesions caused by tobacco mosaic virus and tobacco necrosis virus in bean leaves.

2.7.1.3 Stem resistance

Resistance in current Australian cultivars is based on selection at the stem infection phase of the disease cycle. Therefore, it is important to explore mechanisms which are acting in this part of the plant. Studies in 1972 by Brunin (cited by Roussel *et al.* 1999b) demonstrated that lignification of *B. napus* stems following infection with *L. maculans* was associated with occlusion of vessels in the resistant cv. Sarepta. Hammond and Lewis (1987b) showed that occlusion of stem xylem vessels with lignin was associated with a more intense and earlier wall lignification of vascular parenchyma cells in the partially resistant cv. Jet Neuf compared to the susceptible cv. Primor after infection of the petiole with an A-group isolate of *L. maculans*. This response was correlated with a sequence of host reactions including lignification, cambium formation and callose deposition as well as the containment of hyphae and limitation of stem lesions. Toluidine blue O stained material revealed that lignin was associated with the resistance of oilseed rape stems to *L. maculans* hyphae in other studies (Nathaniels and Taylor 1983; Xi and Morall 1993). Annis and Goodwin (1997a) found that stem extracts from resistant cultivars of canola contained a water-soluble inhibitor of the

polygalacturonase activity of *L. maculans*. Polygalacturonases, produced by the pathogen, degrade pectin in canola cell walls by hydrolysis. Hammond and Lewis (1986b) illustrated this process with transmission electron microscope images of the swollen and lamellate structure of cell walls adjacent to hyphae in stem cankers. Furthermore, Annis and Goodwin (1997a) discovered that there was a significant correlation between activity of this inhibitor and stem resistance to blackleg. In addition, it was also found that calcium levels were positively correlated to the level of inhibition of polygalacturonase activity, possibly linking calcium with resistance (Annis and Goodwin 1997b). Chen and Seguin-Swartz (1999) revealed that when stem tissues of *D. muralis* and *D. tenuifolia* were inoculated with *L. maculans*, the hyphae which colonised the xylem and phloem were embedded in lignin-containing materials and canker did not develop.

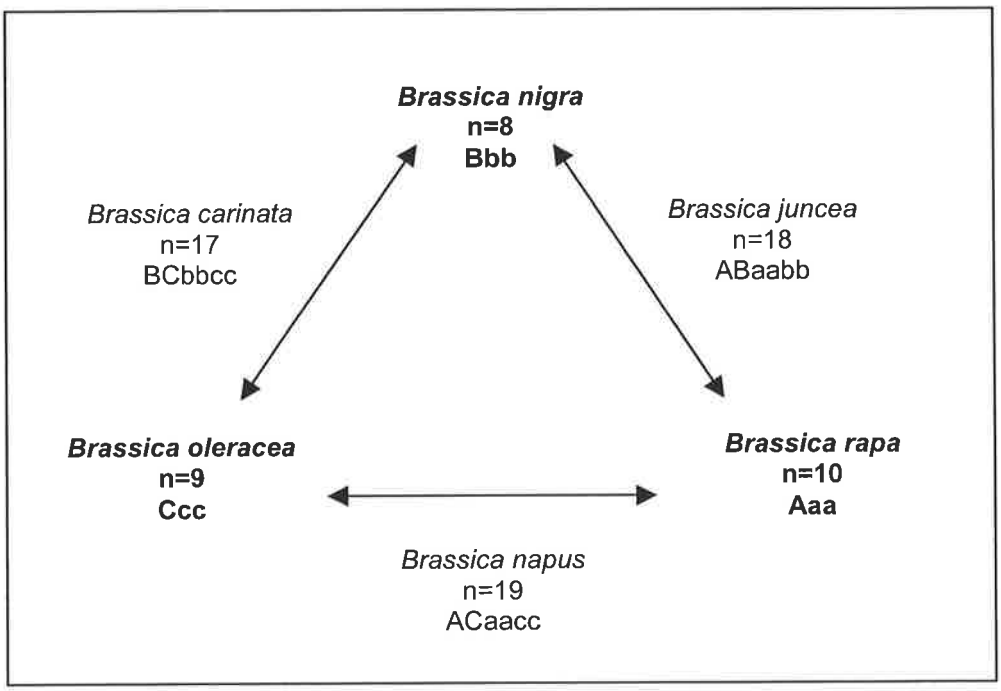
Stem resistance in potato to *Erwinia carotovora* var. *atroseptica* was related to increased tissue calcium and magnesium concentrations (Bain *et al.* 1996). Phytoalexins have been shown to be related to resistance in the roots of chickpea to *Fusarium oxysporum* var. *ciceri* (Stevenson *et al.* 1997). Angelini *et al.* (1993) studied the resistance mechanisms of chickpea stems to *Ascochyta rabiei*, and results suggested that the structural organisation of xylem tissues, polyamine metabolism and peroxidase activity all have a role.

Previous resistance studies have examined canola cultivars which are either used in other countries or are not used in Australia any more. Current Australian cultivars have improved resistance and studies need to be carried out to identify the mechanisms which act to reduce the stem canker phase of blackleg.

2.7.2 Inheritance of resistance

The diploid species, *B. oleracea, B. nigra* and *B. rapa*, were probably the first to be cultivated and then the amphidiploid species, *B. carinata, B. juncea* and *B. napus*, arose when the two parent species were cultivated together (Buzza 1995). Early selections would have been made for seed size and seed retention. All three amphidiploids are self-compatible while parental diploids are self-incompatible, suggesting that self-compatibility may have also been selected for also. The most domesticated groups of *Brassica* species come from areas where these oilseeds have been cultivated for a long time (Buzza 1995). For example, *B. juncea* and *B. carinata* have relatively shatter-resistant pods, whereas the more recent *B. napus* has little resistance to shattering. There is more genetic diversity in ancient species such as *B. rapa* and *B. juncea* than in *B.napus*, which is due to their wider dispersal leading to selection for adaptation to a wider range of environments and for different characteristics. The genomic relationships of oilseed *Brassica* species are illustrated in Figure 2.7.

Figure 2.7: Genomic relationships of oilseed *Brassica* species (taken from Rimmer and van den Berg 1992). n = haploid number of chromosomes, AaBbCc = genomic characterisation of cytoplasm (upper case) & genome (lower case)



The development of winter oilseed rape cultivars, such as Jet Neuf and Rafal in Europe, which had some resistance to blackleg, showed that resistance was a heritable and selectable character. Roy and Reeves (1975) found that 25% of the F_2 population was resistant to blackleg in crosses between blackleg resistant European winter material and susceptible cultivars adapted to Australian conditions. By crossing cv. Major (European blackleg resistant cultivar) with cv. Oro (Australian blackleg susceptible cultivar), Roy (1978b) produced Wesreo, the first Australian cultivar with blackleg resistance.

Roy (1978a) attempted to transfer resistance genes from *B. juncea* (aabb) to *B. napus* (aacc) through interspecific crossing. This work showed that the gene(s) for seedling resistance lie in the B genome, but it was concluded that transferring complete (seedling + adult) resistance to cultivated oilseed rape was unlikely. Six years later, however, Roy (1984) managed to transfer complete resistance to blackleg from *B. juncea* to *B. napus* through an interspecific cross, disproving his earlier suggestion. It was also later shown that *Brassica* species which contain the B genome (*B. nigra, B. carinata* and *B. juncea*) possess a hypersensitive type of resistance to blackleg that is effective throughout the life of the plant (Rimmer and van den Berg 1992).

More successful interspecific crosses which transferred resistance to blackleg into oilseed rape were made by Sacristán and Gerdemann (1986). They produced interspecific crosses between three cultivars of oilseed rape (*B. napus*) and the resistant species *B. juncea* and *B. carinata*. Mithen and Lewis (1988) examined the inheritance of resistance to *L. maculans* in a cross between *B. oleracea* var. *alboglabra* (Chinese Kale) and *B. insularis* (a resistant, wild *Brassica* species). They reported that the dominance of hypersensitive resistance within the F_1, together with the segregation of resistance and susceptibility in the F_2 progeny, suggested that resistance to *L. maculans* is determined by two independently-segregating dominant

genes. This genetic basis would facilitate breeding for resistance into lines of *B. napus* following interspecific crosses with *B. rapa* and subsequent *in vitro* embryo culture.

The effect of A genome substitution on the resistance of *B. napus* to blackleg was studied by Crouch *et al.* (1994). Six accessions from four different subspecies of *B. rapa* were crossed with *B. oleracea* var. *alboglabra* in order to develop a series of synthetic *B. napus* lines with a common C genome but contrasting A genomes. Synthetic lines derived from two wild accessions of *B. rapa* and their F_1 hybrids with oilseed rape cultivars, expressed high levels of resistance in glasshouse experiments. One of these lines, produced from a cross between *B. oleracea* var. *alboglabra* and an accession of *B. rapa* var. *sylvestris* collected from Sicily, also expressed high levels of resistance in field experiments in England and Australia when exposed to a genetically diverse pathogen population. This line was used in the development of the resistant cvs Hyola 60 and Surpass 400 by Pacific Seeds (Buzza and Easton 2002). All other synthetic lines and cultivars were highly susceptible in both glasshouse and field experiments. F_1 hybrids between oilseed rape cultivars and synthetic lines derived from *B. rapa* var. *chinensis* were significantly more susceptible than both parents (Crouch *et al.* 1994). These results suggested that the background resistance in an oilseed rape parent is determined by a recessive gene (or genes) and shows the complex nature of the genetic interaction between host and pathogen.

Chèvre *et al.* (1996) characterised *B. nigra* chromosomes and blackleg resistance in *B. napus-B. nigra* addition lines. A significant effect on the development of stem canker in field conditions was observed only for the line carrying chromosome 4, which was more resistant than the susceptible control. The authors suggested that the presence of the resistance gene in oilseed rape cultivars may induce a change in the structure of *L. maculans* populations, inducing a breakdown of the introduced resistance. Subsequent studies by Chèvre *et al.*

(1997) revealed that the blackleg resistance gene was located on chromosome 8 rather than on chromosome 4. However, they did confirm that the resistance gene is carried by the B genome of *B. juncea*. Struss *et al.* (1996) showed that stem resistance lies in the B-genome for *B. nigra, B. juncea* and *B. carinata,* corresponding with the results of Dixelius and Wahlberg (1999) who found similar results for adult leaf resistance. Somda *et al.* (1999) reported race-specific resistance of oilseed rape to *L. maculans* and suggested that the resistance gene *Jlm 1*, found in the B-genome, although effective against most virulent strains of *L. maculans*, should probably be used in association with other sources of resistance, to delay resistance breakdown.

The potential durability of new sources of resistance was evaluated by Brun *et al.* (2000). They found that the *Jlm*1 gene in recombinant lines MX and MXS maintained a high level of resistance. However, 3 or 4 years after the beginning of the experiment, the resistance of lines exposed to inoculum produced from their own residues broke down. Resistance of the addition line LA4+, containing the *B. nigra* chromosome 4, remained highly resistant to all sources of inoculum for the 4 years of the experiment.

Marcroft *et al.* (2002) screened a range of *Brassica* species to identify alternative sources of blackleg resistance for Australian breeding programs. They concluded that screening for seedling survival would not be useful for producing adult plant resistance and that *Brassica* lines with the B genome, especially *B. carinata*, and the winter *B. napus* types should be used as sources of resistance.

2.8 DISEASE CONTROL

Plant disease results from an interaction of host, pathogen and the environment. Control cannot arise from strategies which target only one of these factors, but rather from an integrated approach (Gugel and Petrie 1992).

2.8.1 Crop rotation

Crop rotation is considered the most important cultural practice available to producers to control blackleg (Kharbanda and Tewari 1996). Severe blackleg can occur when oilseed rape is sown in, or adjacent to, fields containing oilseed rape stubble infested with *L. maculans* (McGee and Emmett 1977; Petrie 1978). Since ascospores can be released from this stubble for as long as the stubble persists (Gabrielson 1983), crop rotation is important. Crop rotation allows time for stubble to decompose in the absence of a susceptible host, removing the source of inoculum. As mentioned in section 2.4.2, this can be as long as 5 years. A 4-year rotation is generally recommended to reduce inoculum of *L. maculans* (Gugel and Petrie 1992; Salisbury *et al.* 1995). Other hosts, such as wild mustard and volunteer oilseed rape, should also be controlled so that they do not act as inoculum sources.

2.8.2 Sanitation

Sanitation is an effective method of controlling blackleg. Clean farm machinery will restrict the movement of stubble-borne plant pathogens from field to field (Kharbanda and Tewari 1996). However, physical removal of infested crop residue is not practical in the field. Burning stubble can be an effective method of sanitation (Gugel and Petrie 1992); however, it has serious environmental implications such as smoke and removal of organic matter from the soil.

2.8.3 Stubble management

The accumulation of large amounts of infested canola stubble can be responsible for high levels of blackleg (Khangura and Barbetti 2001). Several studies have confirmed that stubble reduction can be achieved through certain management strategies. Kharbanda and Ostashewski (1997) found that buried stubbles bore pseudothecia within 6 months, and stubble on the surface developed pseudothecia only after 12 months; however, the buried stubble decomposed at a much greater rate. Likewise, buried canola stubble underwent up to twice as much decomposition as stubble on the soil surface (Blenis *et al.* 1999). Turkington *et al.* (2000a) reported a 40% dry weight reduction in stubble which was incorporated, compared to only 27% dry weight reduction for stubble which was on the soil surface. In another study, Turkington *et al.* (2000b) found that stubble retention was greater for zero tillage than for conventional tillage, and that the rate of decomposition for shoot residues was greater than for root residues. These results confirm that ploughing in the canola stubble and then using shallow minimum tillage or direct seeding to prevent stubble coming back up to the surface can be an effective method of control (Gladders and Musa 1980), as burying the stubble greatly increases the rate of decomposition and thereby reduces inoculum levels rapidly and, furthermore, ascospores cannot be released into the air (MacNish 1979). The rare occurrence of stem canker in China has been attributed to cultural practices that result in the removal of most potential inoculum (West *et al.* 2000).

Petrie (1995a) demonstrated that several herbicides and fungicides, including glyphosate, dinoseb, benomyl, propiconazole and triadimenol, when applied directly to stubble, by immersion for 10 s, which was then placed on soil in pots outside reduced ascospore production by *L. maculans*. However, Turkington *et al.* (2000a) showed that, using commercial spray technologies and application rates in the field, a range of herbicides (including glyphosate) had no significant effect on decomposition of canola stubble or

sporulation of *L. maculans* compared to the distilled water control. These contradictory results may be due to the application of chemicals in the field by Turkington *et al.* (2000a) compared to the application of chemicals in the laboratory by Petrie (1995a).

In Australia, where stubble is very persistent, destruction of infested stubble by raking and burning or burying is presently recommended, and improved methods for management of the stubble are being sought (Barbetti and Khangura 1999; Barbetti and Khangura 2000).

2.8.4 Sowing time

Growers in England can sow winter oilseed rape early so that the plants are well established before ascospore release peaks (Gladders and Musa 1980). This allows them to take advantage of the reduced impact of the disease on plants that are infected at later growth stages (McGee and Petrie 1979). Delayed sowing of oilseed rape crops was an accepted means of control of blackleg in Victoria according to McGee and Emmett (1977), even though there was a yield penalty associated with this practice. This practice was developed from studies by McGee (1977) which showed a decline in the severity of disease in crops sown later in the year, attributed to the presence of fewer ascospores later in the season. In contrast, Khangura and Barbetti (2001) found that sowing date had no effect on disease severity or incidence in Western Australia. The contradiction between these two findings may be due to the different environmental conditions in each area. Climatic conditions leading up to, and early in, the season may have an effect on timing and severity of ascospore infection, highlighting the need for a forecasting system to identify the appropriate sowing time each year.

2.8.5 Resistance

The development of resistant cultivars is by far the most effective method of control against blackleg. Resistance has been identified in germplasm in Australia, Canada and Europe (Cargeeg and Thurling 1980; Rimmer and van den Berg 1992). Some of the most resistant cultivars which are presently available to producers in Australia include *B. napus* cvs Hyola 60, Surpass 400, 402CL and 603CL, Ag-Emblem and Ag Outback (Appendix 1). The introduction of new, more resistant cultivars should be used in conjunction with other management practices to optimise disease control. A continuous breeding effort has to be maintained in order to widen the genetic base of resistance present in oilseed rape cultivars (Gugel and Petrie 1992). This will help to prevent the rapid breakdown of host resistance by the appearance of more virulent strains of the pathogen.

2.8.6 Fungicides

Environmental and health concerns mean that control by chemical fungicides should only be used as a last resort. In Canada and Europe, fungicides such as benomyl, carbathiin, thiram and iprodione can control seed-borne inoculum when used as seed treatments (Rempel and Hall 1995; West *et al.* 2001). Blackleg infection from infested crop residue has been successfully controlled in Australia using flutriafol-coated superphosphate fertiliser (Ballinger *et al.* 1988; Khangura and Barbetti 2001) and this treatment was shown to increase yield (Khangura and Barbetti 2002). This treatment is marketed as Impact® In-Furrow Fungicide and is presently the most common form of fungicide treatment used in the control of blackleg in Australia. However, this is economically viable in some conditions only, for example, when using cultivars of low resistance in higher rainfall areas and when the level of inoculum is high.

The use of foliar fungicides has proved to be ineffective for the control of blackleg stem canker in Australia (Brown *et al.* 1976). However, the application of benomyl, 8 weeks after sowing, reduced the proportion of plants with stem lesions, and the reduction was greater for a more resistant cultivar (Nuzum and Kaldor 1988). Propiconazole has been used as a foliar fungicide in Canada, but does not give complete control of stem canker (Kharbanda *et al.* 1999). In western Europe, difenoconazole alone or mixed with carbendazim or flusilazole plus carbendazim have been effective for the control of crown canker (Gladders *et al.* 1998, cited by West *et al.* 2001). Sun *et al.* (2001) reported that foliar applications of difenoconazole and carbendazim reduced the severity of crown canker at harvest. Foliar spraying with triazole compounds such as hexaconazole, tebuconazole, triadimefon, triadimefon, diconazole and uniconazole has also been reported to control blackleg (Rempel and Hall 1995).

The efficacy and economics of fungicide controls are reliant on a range of factors such as climate and other management strategies, especially in Australia where potential yields are less than in Europe. This further highlights the need for an efficient disease forecasting system in this country, which can only be achieved once sufficient epidemiological data have been collected.

2.9 SUMMARY

Blackleg is an important disease of canola. The rapid expansion of the industry in Australia has increased the risk of disease on the crop by increasing inoculum levels. Many mechanisms contribute to the overall resistance which some canola cultivars display against the blackleg fungus. The leaf infection stage has been the focus of most studies in the past. Resistance in current cultivars appears to occur predominantly in the stem and petiole region,

due to selection for resistance to stem canker by canola breeders, so it is important to gain more information about the mechanisms which act in this region of the plant.

The optimal conditions for infection of canola by *L. maculans* have been determined through many controlled environment and field experiments. However, much of this information has been compiled using cultivars of canola grown in countries other than Australia, or Australian cultivars which are no longer commercially available. There is a need for more knowledge on the effects of local environmental conditions on the potential for epidemics to occur on a range of currently available cultivars. This would help growers to select the best cultivars and management strategy for their situation.

Survival of the blackleg fungus is still not completely understood. Recently, molecular DNA-based technology has provided sensitive and efficient tools for disease diagnosis in a range of crops. When developed specifically for *L. maculans*, this technique should allow the evaluation of soil and stubble samples and enable investigation of the survival of the fungus and the potential infectivity of soil and stubble. This information will assist growers in the management of blackleg.

2.10 RESEARCH OBJECTIVES

The objectives of this research were to:

- Investigate the mechanisms of blackleg resistance in the leaf, stem and petiole regions of canola cultivars using scanning electon microscopy and histochemical methods.
- Examine the epidemiology of *L. maculans* in canola and model the effects of environment and cultivar on the development of blackleg in the field.

- Validate a DNA-based assay for evaluating soil and stubble populations of the blackleg fungus, study the survival of the pathogen and determine the effects of cropping practices on disease carryover.

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 PLANTS

3.1.1 Canola cultivars

Canola seed was supplied mainly by Mr Trent Potter, South Australian Research and Development Institute (SARDI), Struan, South Australia, except for cv. Hyola 60, which was supplied by Mr Andrew Easton, Pacific Seeds and doubled haploid cvs Scoop and Westar, which were supplied by Mr Neil Wratten, New South Wales Agriculture. The cultivars listed in Table 3.1 were used for experiments in this project. Blackleg ratings and status were determined using the blackleg resistance rating system developed by the Canola Association of Australia (CAA) and based on Australian Crop Accreditation System (ACAS) protocols (Marcroft 2001).

Table 3.1: Maturity and blackleg status of canola cultivars used in experiments (taken from Potter and Stanley 2002).

Cultivar	Maturity	Blackleg rating*	Blackleg status
Hyola 60	mid	9	highly resistant
Dunkeld	mid-late	6	resistant
Mystic	early	6	resistant
Scoop	mid	6	resistant
Oscar	mid	6	resistant
Pinnacle (TT)	mid-late	5.5	moderately resistant
Monty	early	5.5	moderately resistant
Karoo (TT)	early-mid	3.5	moderately susceptible
Hyola 42	early	2	susceptible
Q2[#]	mid-late	2	susceptible
Westar[#]	mid-late	1.5	susceptible

TT = triazine tolerant

*1 is most susceptible, 9 is most resistant,

[#] Q2 and Westar are Canadian cultivars that are very susceptible in Australian variety trials

3.1.2 Plant growth

3.1.2.1 Potting soil

The potting soil used in all glasshouse experiments was a recycled soil produced by Plant Growth Services, Plant Research Centre, Waite Campus, as follows. Half a cubic metre of composted experimental peatmoss/sand potting mix and plants therein (composted for over 2 years) was steam sterilised at 100°C for 45 minutes. On cooling, one tenth of a cubic metre of Eurotorf® peatmoss was incorporated and then the following nutrients were added:

Blood meal	500g
Potassium sulphate	200g
Agricultural lime	200g
Super phosphate	100g

The mix, which had a pH of approx. 6.5, was sieved through a 1cm sieve. Soil was stored for less than 1 month in plastic bins, in a shaded, cool, enclosed area prior to use.

3.1.2.2 Plant maturity

A growth-stage (GS) key devised by Harper and Berkenkamp (1975), described in Appendix 2, was used to refer to plant maturity.

3.1.2.3 Glasshouse conditions

The glasshouse used in experiments was 18.5 m x 9.5 m x 2.9 m and constructed with Lexen® 10 mm twin walled poly-carbonate sheeting and maintained at 18-29°C with evaporative cooling and convection heating in near natural daylight conditions (10% UV filtered out). On benches, plants were watered by hand once per day with tap water.

3.2 FUNGI

Isolates of *L. maculans* were selected from a South Australian collection maintained by the Pulse and Oilseed Pathology group, SARDI since 1997 (Table 3.2). Methods of collection are described in section 3.2.1. Isolates were classified as A-group (virulent) or B-group (avirulent) according to cultural characteristics, pathogenicity tests and confirmed by PCR analysis (Sosnowski *et al.* 2001b).

Table 3.2: South Australian isolates of *L. maculans* used in this project, their origin and date of collection.

Isolate	Origin	Date collected	Classification
66/97	Millicent	4/8/1997	A-group
119/97	Kadina	28/8/1997	A-group
120/97	Two Wells	28/8/1997	B-group
213/97	Minnipa	5/11/1997	B-group
165/98	Charlick RS	16/9/1998	A-group
126/99	Kingsford RS	9/9/1999	A-group
127/99	Charlick RS	9/9/1999	A-group
208/00	Bordertown	15/11/2000	A-group

RS – Research Station

3.2.1 Collection and storage of *L. maculans* isolates

3.2.1.1 Culture media

The following media were used routinely for culturing isolates of *L. maculans*; water agar, potato dextrose agar (PDA) and quarter strength potato dextrose agar (¼ PDA), as described in Appendix 3. The potential of ¼ PDA as a selective medium was evaluated (see section 7.2.1).

3.2.1.2 Establishment of isolates from pycnidiospores

Leaf and stem samples were collected from infected plants in commercial canola crops. Tissue with lesions was excised from the plants in the laboratory and surface sterilised in a laminar flow cabinet in 100% ethanol, then 100% Milton bleach solution (1% available chlorine) and, finally, rinsed in sterilised reverse osmosis (RO) water, 30 s in each, and then

left on sterile filter paper to dry. Small sections of lesion with pycnidia (no more than 5 mm^2) were excised and plated on ¼ PDA. The cultures were incubated at room temperature (approximately 22°C) for 2 weeks under blacklight (Hitachi 8W/BL350) and fluorescent light (Phillips 18W/W43) for 12 h each day. Single spore-derived cultures were prepared by flooding 2-week-old cultures with sterile RO water and releasing spores by gently rubbing the culture surface with a bent glass rod. The spore suspension was streaked onto water agar with a wire loop and incubated under blacklight and fluorescent light. After 48 h, germinating single spores were located through a Leica MS5 binocular microscope at 50x magnification, in a laminar flow cabinet. It was observed that almost 100% of pycnidiospores germinated on water agar. Single spores were scooped from the water agar with a sterile dental implement with a bent scoop on the end and cultured on ¼ PDA. The cultures were incubated under blacklight and fluorescent light as above for a further 2 weeks and then 3 mm diameter plugs were removed from all areas of one culture derived from a single spore, using a cork borer, and stored in sterile RO water at 3-4°C.

3.2.1.3 Establishment of isolates from ascospores

Stubble with pseudothecia, infected in the previous season, was collected from fields and rinsed under tap water. Areas of the tissue covered with pseudothecia were cut into 5 mm^2 sections and then surface sterilised by first soaking in 100% ethanol for 30 s and then 100% Milton bleach solution (1% available chlorine) for a further 60 s. The samples were soaked in sterile RO water for 3 min, dried on filter paper for 5 min, then attached by means of vaseline to the lids of 9 cm diameter Petri dishes containing water agar. The moist pseudothecia released ascospores, which landed on the water agar. Two days later single germinating ascospores were removed from the agar using a sterile scalpel, then plated on fresh ¼ PDA and incubated under blacklight and fluorescent light. Plugs, 3 mm diameter, were removed and stored in sterile RO water at 3-4°C, as above.

3.2.1.4 Preparation of pycnidiospore suspension

Isolates were cultured from stored plugs on ¼ PDA for 2-3 weeks under blacklight and fluorescent light to produce pycnidiospores. The plates were flooded with 10 ml of sterile RO water. With a bent glass rod, the surface of the cultures was gently rubbed to suspend the pycnidiospores. The suspension was poured into a sterile beaker. The concentration of pycnidiospores was determined with a haemocytometer, and adjusted to approximately 10^6 spores/ml sterile RO water. Tween 20 (BDH Laboratory Supplies, England) was added as a surfactant at 0.05%.

3.3 STATISTICAL ANALYSIS

Analysis of variance (ANOVA) and linear regressions were performed on data from experiments using Statistix for Windows™ (version 2). To check homogeneity of variance, scatter plots of residual versus fitted values were produced and normal distribution was tested using Wilk-Shapiro rankit plots (Anonymous, 1994b).

CHAPTER 4

EXPLORING MECHANISMS OF RESISTANCE

4.1 INTRODUCTION

L. maculans ascospores and pycnidiospores attach to the surface of the leaf, germinate, and produce hyphae, which penetrate the epidermis through stomata and wounds to colonise the mesophyll cells (Hammond and Lewis 1987a; Chen and Howlett 1996; Sosnowski *et al.* 2001b). As discussed in section 2.7.1.2, past studies have revealed that resistance mechanisms at this early stage of infection include; hyphal disintegration in the presence of heavily stained unidentified material (Xi and Morrall 1993), rapid necrosis of the guard cell causing hyphal arrest and callose accumulation in walls of mesophyll cells (Chen and Howlett 1996), hyphal restriction due to reinforcement of cell wall barriers, including wall apposition, papillae and vessel plugging (Roussel *et al.* 1999a), and a reduced density of stomatal pores which decreased the frequency of hyphal penetration (Sosnowski *et al.* 2001b).

The hyphae then grow down the petiole in the xylem vessels or between cells of the xylem parenchyma and cortex (Hammond *et al.* 1985). The fungus then invades and kills cells of the stem cortex, resulting in a canker that may completely girdle the base of the stem (Salisbury *et al.* 1995). Resistance in current Australian cultivars is based on selection at the stem infection phase of the disease cycle. Mechanisms of resistance in the stem include; lignification and callose deposition of the xylem vessels (Hammond and Lewis 1987b; Chen and Seguin-Swartz 1999), inhibition of polygalacturonase activity of the fungus and increased calcium levels (Annis and Goodwin 1997a).

The Scanning Electron Microscope (SEM) has been used to observe *L. maculans* in oilseed rape (Hammond *et al.* 1985) and in Indian mustard (Chen and Howlett 1996). Many other

fungal-plant interactions have been examined using SEM (Angelini 1993; Benhamou and Chet 1996; Benhamou and Chet 1997; Roderick and Thomas 1997; Dushnicky *et al.* 1998; Shankar *et al.* 1998; Smith *et al.* 1999). The SEM works by scanning an electron beam across the surface of a specimen, magnifying up to 100,000 times, compared to the 1,000 times maximum magnification of light microscopy (Terlet 1992). It also has a much greater depth of field than the optical light microscope, so that rough surfaces can be kept in focus. The Field Emission Scanning Electron Microscope (FESEM) was utilised for these experiments as it produces higher quality and better image resolution than the conventional SEM (M. Henderson, personal communication, 2000).

Various compounds have been associated with plant defence mechanisms because of their general occurrence at infection sites (Hare 1966). Lignification is the process in which plant cell walls and intercellular spaces are impregnated with lignin, causing a thickening of the cell walls (Dushnicky et al. 1998). This forms a barrier to the spread of hyphae of most plant pathogens, which are not capable of degrading lignin or lignified cell walls. A number of reagents can be used to stain host material for detection of lignin using the light microscope, including toluidine blue O and phloroglucinol-HCl (Vance *et al.* 1980; Hammond and Lewis 1987b; Southerton and Deverall 1990; Mouzeyar 1993; Angra-Sharma 1994; Dai *et al.* 1995; Dushnicky *et al.* 1998). Callose and suberin can produce a barrier in the same way as lignin, and are detectable using aniline blue and Sudan IV stains, respectively (Jensen 1962; Faulkner and Kimmins 1975; Hammond and Lewis 1987b; Southerton and Deverall 1990; Angra-Sharma 1994; Dai *et al.* 1995; Tynan *et al.* 1998; Jabaji-Hare 1999).

Interactions between *L. maculans* and seven Australian cultivars of canola were examined using the FESEM and histochemical staining techniques. Comparisons were made between resistant and susceptible cultivars with the aim of identifying mechanisms of resistance.

4.2 MATERIALS AND METHODS

4.2.1 Scanning electron microscopy

4.2.1.1 Glasshouse experiments

Experiment 1

Doubled haploid canola seed of cvs Scoop (moderately resistant to blackleg) and Westar (susceptible) was supplied by Mr Neil Wratten. The experiment consisted of six trays (three trays per cultivar), each containing nine punnets (900 ml). Each punnet was filled with potting soil (section 3.1.2.1) and sown with two seeds, later thinned to one plant. Plants, at GS 2.3, were inoculated by placing many 10 µl droplets of pycnidiospore suspension (10^6 spores/ml, section 3.2.1.4) of *L. maculans* isolate 66/97 (A-group) and 120/97 (B-group) on the adaxial surface of leaves using an Eppendorf Multipette® plus (Figure 4.1A). One tray of each cultivar was inoculated with each isolate. Controls consisted of one tray of each cultivar treated as above with 10 µl droplets of sterile RO water with 0.05% Tween surfactant. Trays with plants were covered with plastic bags for 4 days to promote high humidity (Figure 4.1B), and then they were left on a bench in the glasshouse (section 3.1.2.3).

Plants were removed at 7, 13, 14, 17, 18, 21, 23 and 28 d after inoculation. Leaf discs were removed using a cork borer (5 mm diameter) from sites where droplets had been placed (Figure 4.1C). Sections (approximately 10 mm) of petiole and stem, with and without symptoms, were cut with a scalpel (Figure 4.1D). Leaf discs and stem and petiole sections were immersed in gluteraldehyde fixative (Appendix 4) and stored at 3-4°C for up to 6 weeks before preparation for FESEM.

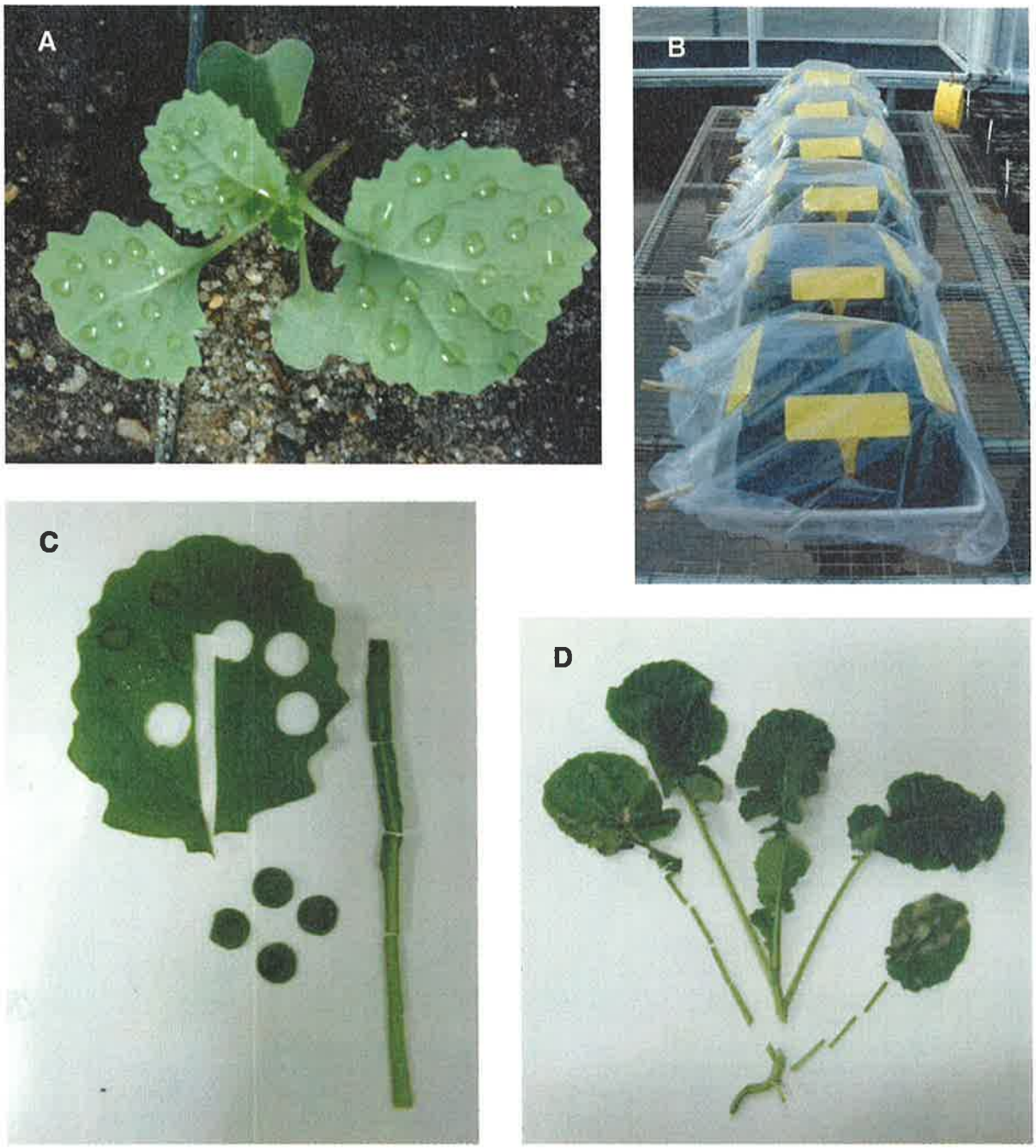


Figure 4.1: A. Inoculation of leaves of canola plants with droplets of pycnidiospore suspension in 0.05% Tween, B. Trays with inoculated plants covered in clear plastic bags on a bench in the glasshouse, C. Leaf discs (5 mm diameter) removed from inoculation droplet sites with a cork borer and petiole sections made with a scalpel and D. Petiole and stem sections made with a scalpel.

Experiment 2

Two canola cultivars were examined; Hyola 60, which is highly resistant to blackleg and Q2, which is susceptible to blackleg (Table 3.1). The experiment consisted of two trays (one tray per cultivar), each containing nine punnets (900 ml). Each punnet was filled with potting soil

and sown with three seeds, later thinned to two plants. Plants, at GS 2.3, in both trays were inoculated by placing many 10 µl droplets of pycnidiospore suspension (10^6 spores/ml, section 3.2.1.4) of the *L. maculans* isolate 66/97 (A-group) on the adaxial surface of leaves using an Eppendorf Multipette® plus. Trays with plants were covered with plastic bags and maintained as described above.

Plants were removed 7 and 14 days after inoculation. Discs were removed from droplet sites on both leaves and cotyledons using a cork borer (5 mm diameter). Leaf and cotyledon discs were immersed in gluteraldehyde fixative and stored at 3-4°C for up to 4 weeks before preparation for FESEM.

4.2.1.2 Specimen preparation for FESEM

Methods for preparation of specimens were based on the Centre for Electron Microscopy and Microstructure Analysis (CEMMSA) laboratory manual for biological scanning electron microscopy (Henderson 2000). Immediately before the dehydration process began, specimens were removed from gluteraldehyde fixative and placed into plastic, screw-top specimen tubes (50 mm x 15 mm diameter) and then agitated twice (10 min each) in washing buffer (phosphate buffered saline, PBS + 4% sucrose, see Appendix 4) on a rotary mixer at 10 rpm. They were then post-fixed in 2% osmium tetroxide in PBS for 1 h on a rotary mixer.

Dehydration

After removing excess osmium tetroxide in solution with a plastic transfer pipette (1 ml), specimens were subjected to a series of washes in acetone, each time placing them on the rotary mixer at 10 rpm. The series comprised; 70% acetone, three changes for 20 min each; 90% acetone, three changes for 20 min each; 95% acetone, three changes for 20 min each; 100% acetone, three changes for 20 min each; 100% acetone, once for 30 min.

Critical point drying

Dehydrated specimens were subjected to critical point drying in a Bal-Tec CPD 030 drier (Henderson 2000).

Specimen mounting and coating

Once dried, the specimens were carefully attached to aluminium mounts using adhesive tabs. Petiole and stem specimens were dissected with a razor blade for cross-sectional and longitudinal views. Specimens were then coated with a thin layer of carbon and gold using a High Vacuum Evaporator (DV-502, Denton Vacuum Inc.).

4.2.1.3 Specimen examination

Specimens were examined and images were acquired using the Philips XL30 Field Emission Scanning Electron Microscope at CEMMSA, University of Adelaide, South Australia.

4.2.2 Histochemistry

4.2.2.1 Glasshouse experiment

Three canola cvs were selected to represent a cross-section of blackleg resistance; Hyola 60 (resistant), Pinnacle (moderately resistant) and Q2 (susceptible). The experiment consisted of six trays (two trays per cultivar), each containing nine punnets (900 ml). Each punnet was filled with potting soil (section 3.1.2.1) and sown with two seeds, later thinned to one plant. Plants, at GS 2.3, were inoculated by placing many 10 µl droplets of pycnidiospore suspension (10^6 spores/ml, section 3.2.1.4) of the *L. maculans* isolate 66/97 (A-group) on the adaxial surface of leaves using an Eppendorf Multipette® plus. Controls consisted of plants treated with 10 µl droplets of sterile RO water with 0.05% Tween surfactant. One tray of each cultivar was inoculated with isolate 66/97 and the other was the control. Trays were

covered with plastic bags for 4 days and maintained in the glasshouse as described in section 4.2.1.1.

Three plants were taken at random from each of the trays 11 days after inoculation and, using a cork borer, 5 mm discs were removed from droplet sites on the leaves (Figure 4.1C). Twenty-eight days after inoculation, two plants were taken at random from each tray for assessment of stems. The first 3 cm of the stem above the crown were removed and placed into a wedge cut out of the side of a carrot. Then cross-sections were made by hand using a razor blade, with sections being stored briefly in sterile RO water to prevent dehydration.

4.2.2.2 Histochemical staining

Fresh leaf discs were cleared of chlorophyll by boiling in 95% ethanol for 5 min, and then placed into 50% ethanol. Stem sections were taken directly from the water and placed into 50% ethanol. Histochemical stains were applied as follows:

Sudan IV (Ruzin 1999)

Leaf and stem specimens were transferred into a tea strainer, keeping treatments separate, and then placed into a glass Petri dish (50 mm diameter) containing Sudan IV solution, which comprised 100 ml of 70% ethanol and 0.07 g of Sudan IV (Sigma), for 10 min. The specimens were then rinsed in 50% ethanol for 2 min in another glass Petri dish. Specimens were then mounted in 50% glycerine on microscope slides for viewing with a light microscope. Red-brown staining indicates the presence of suberin.

Toluidine blue O (Hammond and Lewis 1987b)

Leaf and stem specimens were transferred into a tea strainer, keeping treatments separate, and then placed into a glass Petri dish (50 mm diameter) containing toluidine blue O solution, which comprised 100 ml of PBS and 0.05 g of toluidine blue O (Aldrich), for 1 min. The

specimens were then rinsed in RO water as above and mounted in 50% glycerine. Blue-green staining indicates the presence of lignin.

Phloroglucinol-HCl (Ruzin 1999)

Leaf and stem specimens were transferred into a tea strainer, keeping treatments separate, and then placed into a glass Petri dish (50 mm diameter) containing phloroglucinol-HCl solution, which comprised 100 ml of 95% ethanol, 16 ml of HCl (concentrated) and 0.1 g of phloroglucinol (May & Baker), for 6 min. The specimens were then rinsed in 50% ethanol as above, and mounted in 50% glycerine. Red-brown staining indicates the presence of lignin.

Aniline blue (Faulkner and Kimmins 1975)

Stem specimens were transferred into a tea strainer, keeping treatments separate, and then placed into a glass Petri dish (50 mm diameter) containing aniline blue solution, which consisted 100 ml of 50% ethanol and 5 g of aniline blue (Gurr, BDH), for 5 min. The specimens were then rinsed in 95% ethanol as above and mounted in 50% glycerine. Blue-green staining indicates the presence of callose.

4.2.2.3 Specimen examination

All mounted specimens were examined using an Olympus Light Microscope BH-2 with 12V 100W halogen lamp. Images were taken through the microscope eyepiece using a Nikon Coolpix 950 digital camera.

4.2.3 Progress through the petiole

The cvs selected for this experiment were Dunkeld (resistant) and Hyola 42 (susceptible). The experiment consisted of two trays (one tray per cultivar), each containing 12 punnets (550 ml). Each punnet was filled with potting soil (section 3.1.2.1) and sown with two seeds, later thinned to one plant. Plants, at GS 2.3, were inoculated by placing many 10 µl droplets

of pycnidiospore suspension (10^6 spores/ml see section 3.2.1.4) of the *L. maculans* isolate 66/97 (A-group) on the adaxial surface of leaves using an Eppendorf Multipette® plus. Trays with plants were covered with plastic bags for 4 days and maintained in the glasshouse as described in section 4.2.1.1.

Petioles with lesions were removed from canola plants 13 and 17 days after inoculation. Petiole sections were surfaced sterilised in a laminar flow cabinet using the following procedure; 100% ethanol for 30 s, 100% Milton bleach solution (1% available chlorine) for 30 s and rinsed with sterilised RO water for 30 s. Using a scalpel, an incision was made at the front edge of the necrotic region and then every 2 mm along the length of the petiole. Sections were placed onto ¼ PDA and incubated at approximately 22°C under blacklight (Hitachi 8W/BL350) and fluorescent light (Phillips 18W/W43) for 5 days before assessment. The presence of *L. maculans* in successive sections was recorded; identification was based on morphological characteristics (Punithalingam & Holliday 1972).

4.3 RESULTS

4.3.1 Scanning electron microscopy

In experiment 1, pycnidiospores on the adaxial leaf surface of cvs Scoop and Westar, started to form germ tubes within 7 days of inoculation (Figure 4.2). Figure 4.3 shows more advanced hyphal growth 7 days after inoculation. Hyphae extended across the leaf surface and penetrated through stomata in several locations. Sometimes hyphae on both cultivars appeared to recognise the stomata, grow towards them and penetrate into the mesophyll (Figure 4.4), however, in many cases the hyphae grew across the opening of the stomata without penetrating them (Figure 4.5).

On the leaf surface, hyphae were associated with tearing of the cuticle (Figure 4.6), which often resulted in contraction of large areas of the epidermis to expose the palisade parenchyma cells of the mesophyll (Figure 4.7). Hyphae entered the mesophyll through these sites of epidermal tearing (Figure 4.8). Epidermal damage was only observed in cv. Westar when inoculated with *L. maculans* pycnidiospores.

In cv. Westar, hyphae were first observed in the xylem vessels of the petiole 13 days after inoculation (Figure 4.9). Hyphae were first observed growing in xylem vessels along the petiole towards the stem in cv. Scoop, 17 days after inoculation, and were regularly found in xylem vessels of the petiole 28 days after inoculation (Figure 4.10). Hyphae had colonised the cells of the cortex near the vascular tissue in both cultivars, 28 days after inoculation (Figure 4.11). Examination of the stem/petiole junction failed to reveal the presence of hyphae in either cultivar. However, hyphae were observed in the stem of cv. Westar, 23 days after inoculation (Figure 4.12), in both the xylem vessels and the cells of the cortex. No hyphae were observed in the stem of cv. Scoop.

In experiment 2, a stringy, adhesive compound was observed between the pycnidiospores and the waxy cuticle layer on the leaf epidermis of cv. Q2 (Figure 4.13). Fourteen days after inoculation with the A-group *L. maculans* isolate 66/97, hyphae had grown profusely over the surface of the susceptible cv. Q2, forming a mycelial mat (Figure 4.14). However, on the highly resistant cv. Hyola 60, 14 days after inoculation with the same isolate, pycnidiospore germination was inhibited and hyphal growth appeared severely restricted (Figure 4.15).

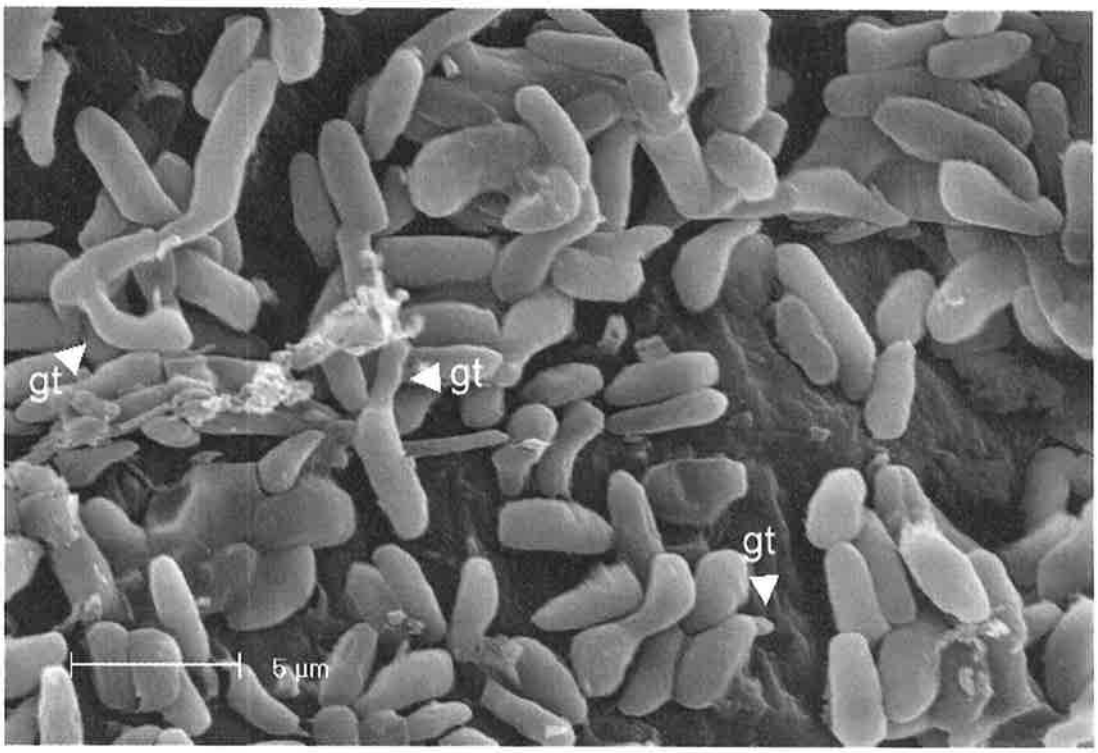


Figure 4.2: Scanning electron micrograph of *L. maculans* pycnidiospores on the adaxial leaf surface of the blackleg resistant canola cv. Scoop, 7 days after inoculation with A-group isolate 66/97. Pycnidiospores are swollen and some have germ tubes (gt).

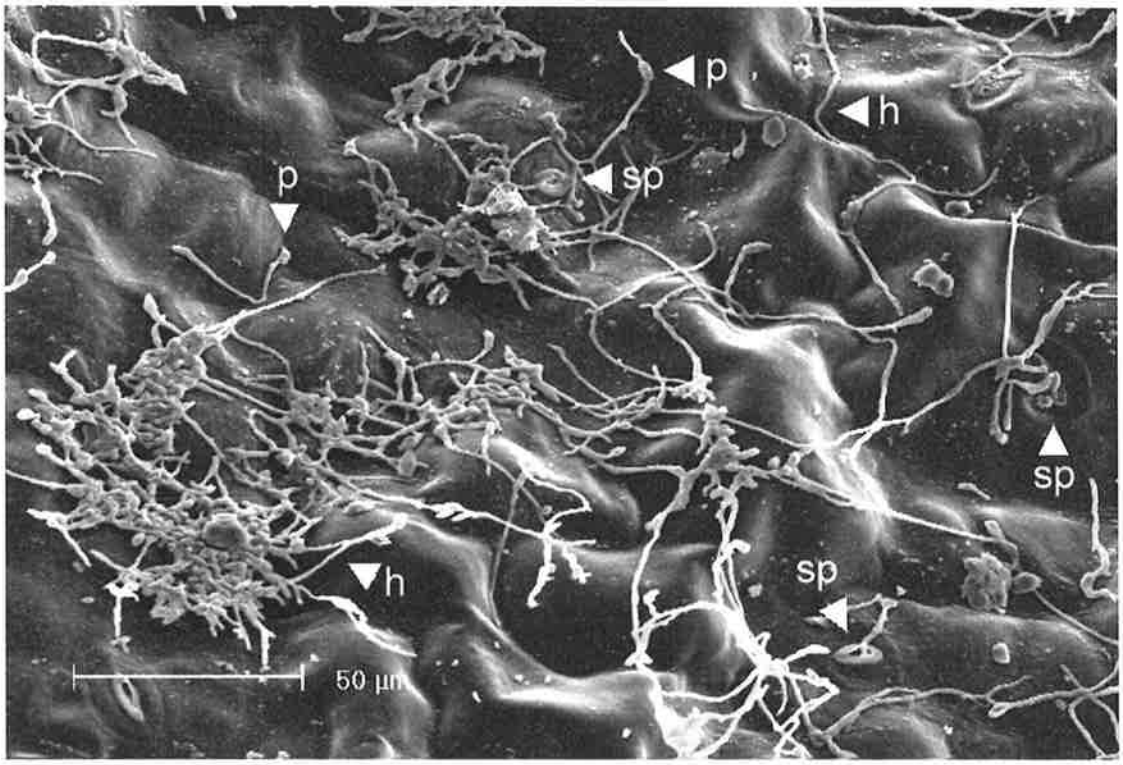


Figure 4.3: Scanning electron micrograph of *L. maculans* pycnidiospores on the adaxial leaf surface of the blackleg resistant canola cv. Scoop, 7 days after inoculation with A-group isolate 66/97. Pycnidiospores (p) have given rise to hyphae (h) which are growing along the epidermis. Some hyphae have penetrated the stomata (sp).

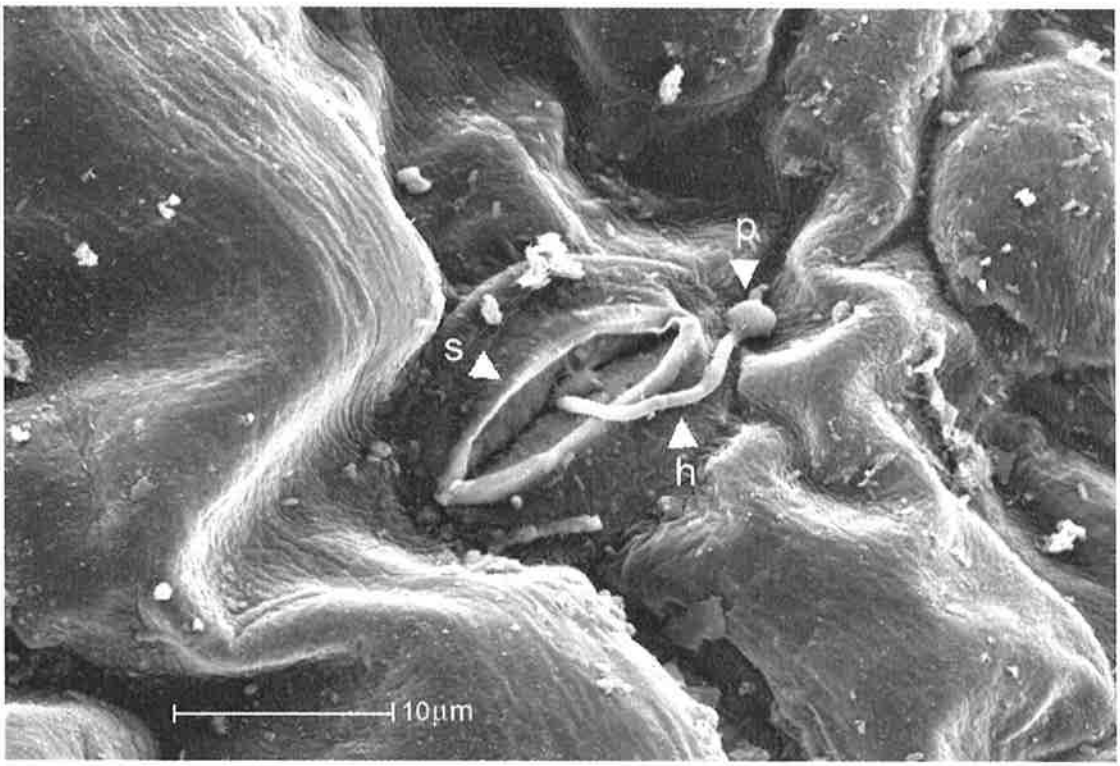


Figure 4.4: Scanning electron micrograph of a *L. maculans* pycnidiospore on the adaxial leaf surface of the blackleg susceptible canola cv. Q2, 7 days after inoculation with A-group isolate 66/97. A hypha (h) extends from the pycnidiospore (p) to penetrate a stoma (s).

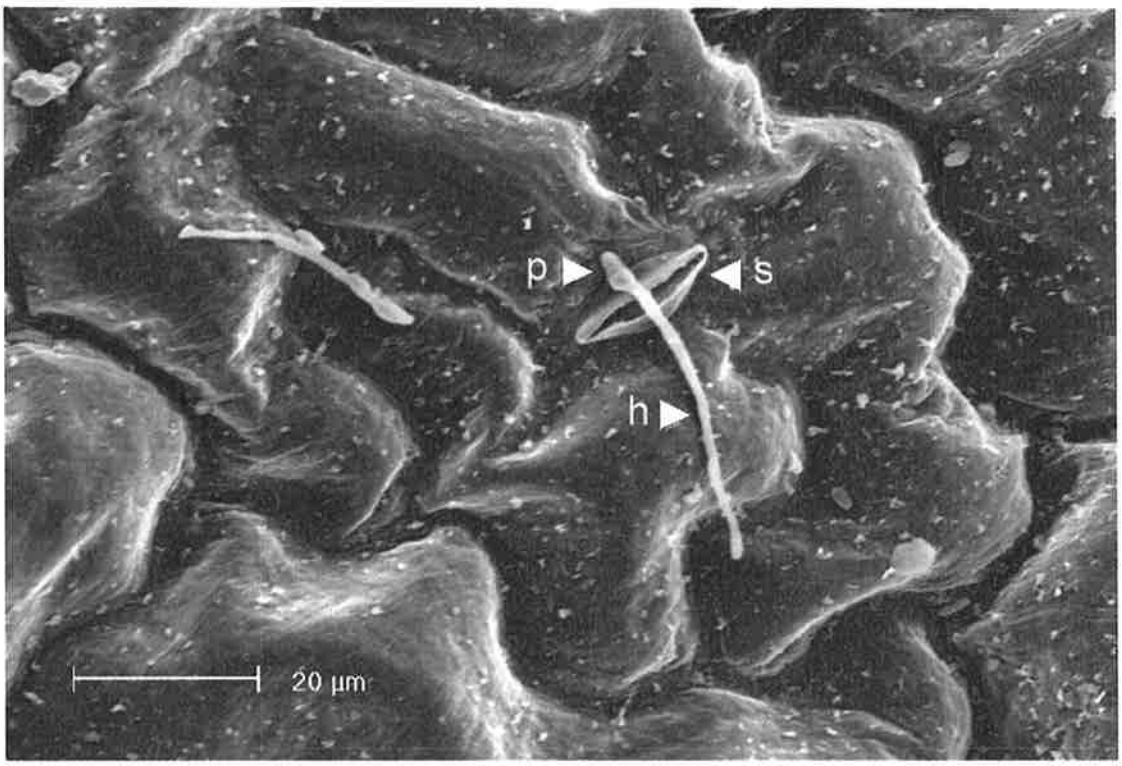


Figure 4.5: Scanning electron micrograph of a *L. maculans* pycnidiospore on the adaxial leaf surface of the blackleg susceptible canola cv. Westar, 7 days after inoculation with A-group isolate 66/97. A hypha (h) extends from the pycnidiospore (p) over the stoma (s) without having penetrated it.

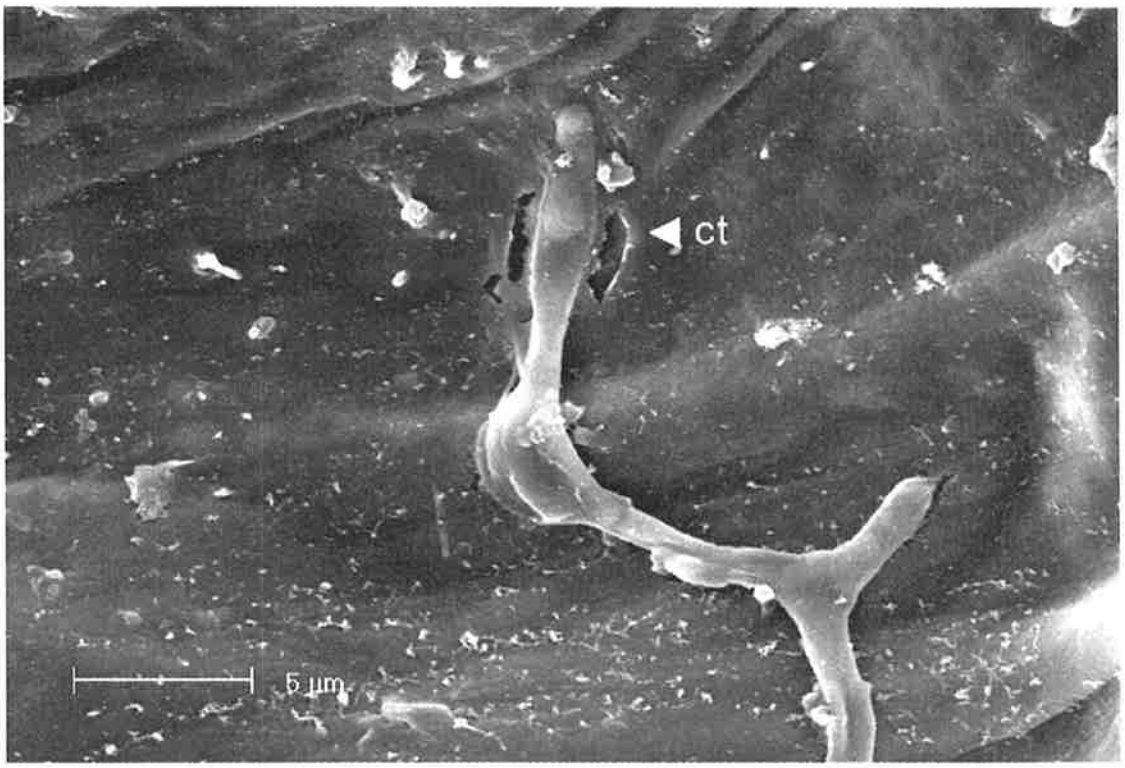


Figure 4.6: Scanning electron micrograph of a *L. maculans* hypha on the adaxial leaf surface of the blackleg susceptible canola cv. Westar, 14 days after inoculation with A-group *L. maculans* isolate 66/97. The hypha is associated with tearing of the cuticle (ct).

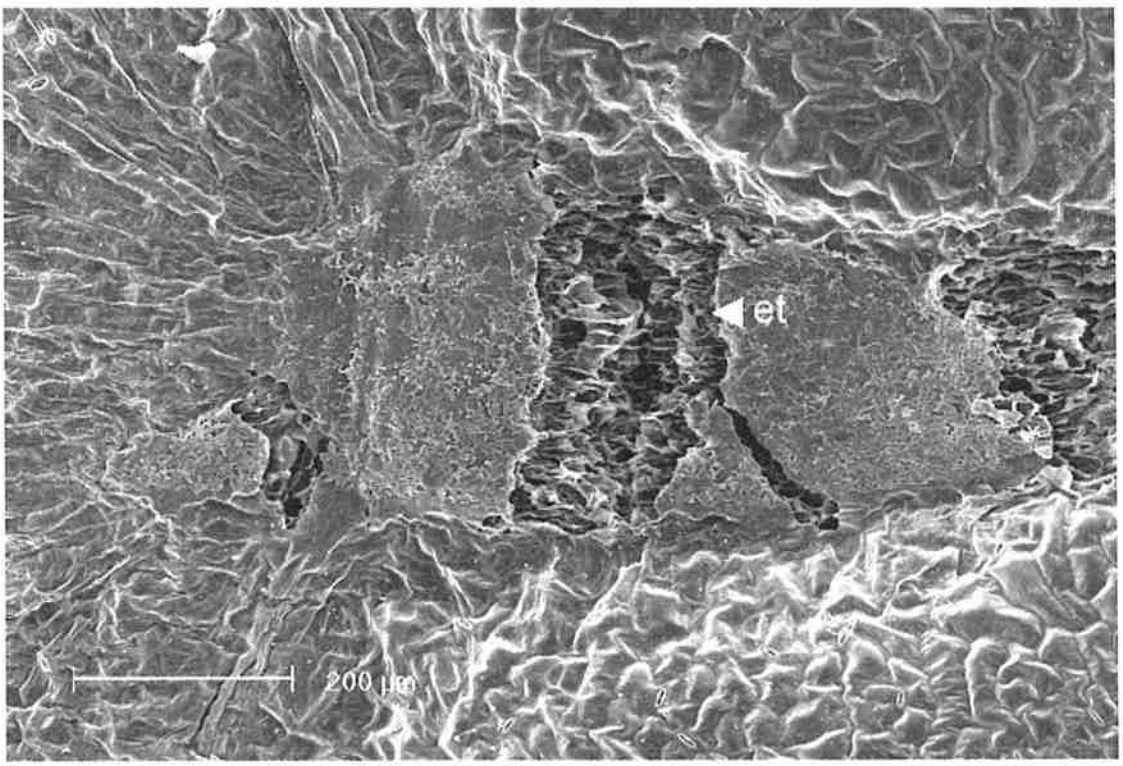


Figure 4.7: Scanning electron micrograph of an inoculation site on the adaxial leaf surface of the blackleg susceptible canola cv. Westar, 14 days after inoculation with A-group *L. maculans* isolate 66/97. Rupture of the cuticle and epidermis (et) occurs in association with deformation of the cuticle in areas of hyphal growth.

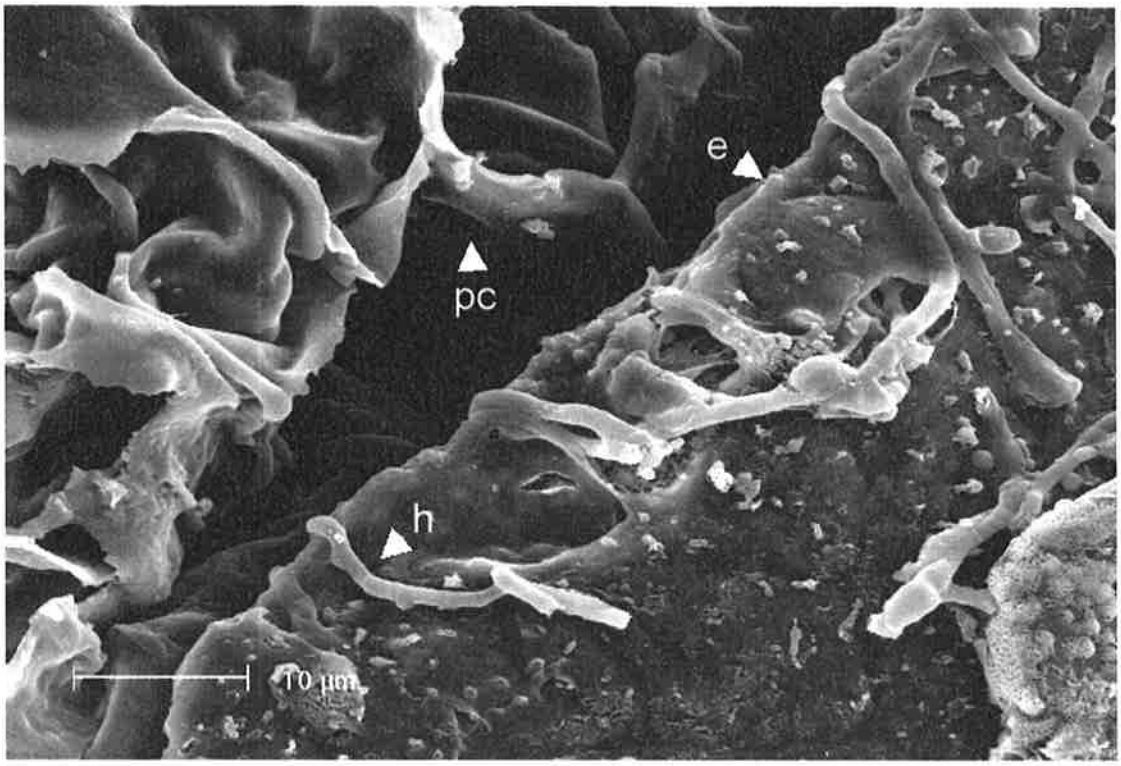


Figure 4.8: Scanning electron micrograph of an inoculation site on the adaxial leaf surface of the blackleg susceptible canola cv. Westar, 14 days after inoculation with A-group *L. maculans* isolate 66/97. Rupture of the cuticle and epidermis allows hyphae (h) to enter the palisade cells (pc) over the edge of the break in the epidermis (e).

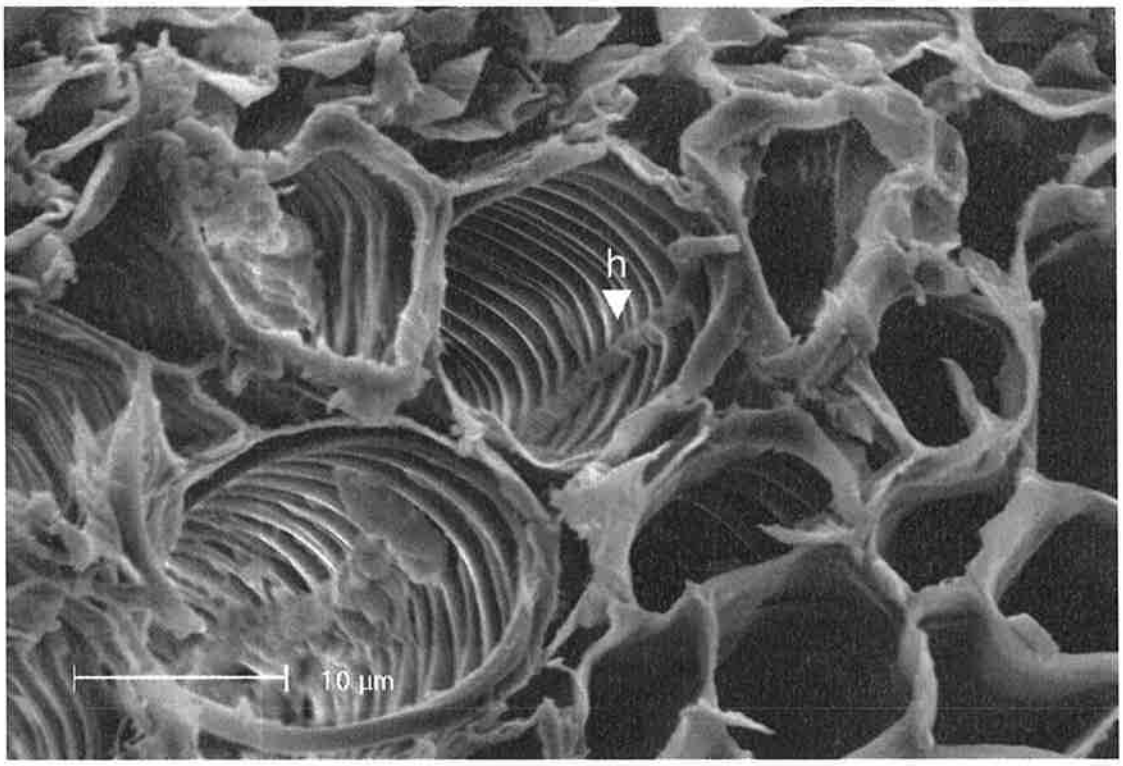


Figure 4.9: Scanning electron micrograph of a cross-section of the petiole of the blackleg susceptible canola cv. Westar, 13 days after inoculation with A-group *L. maculans* isolate 66/97. A hypha (h) extends through the xylem vessel from the leaf towards the stem.

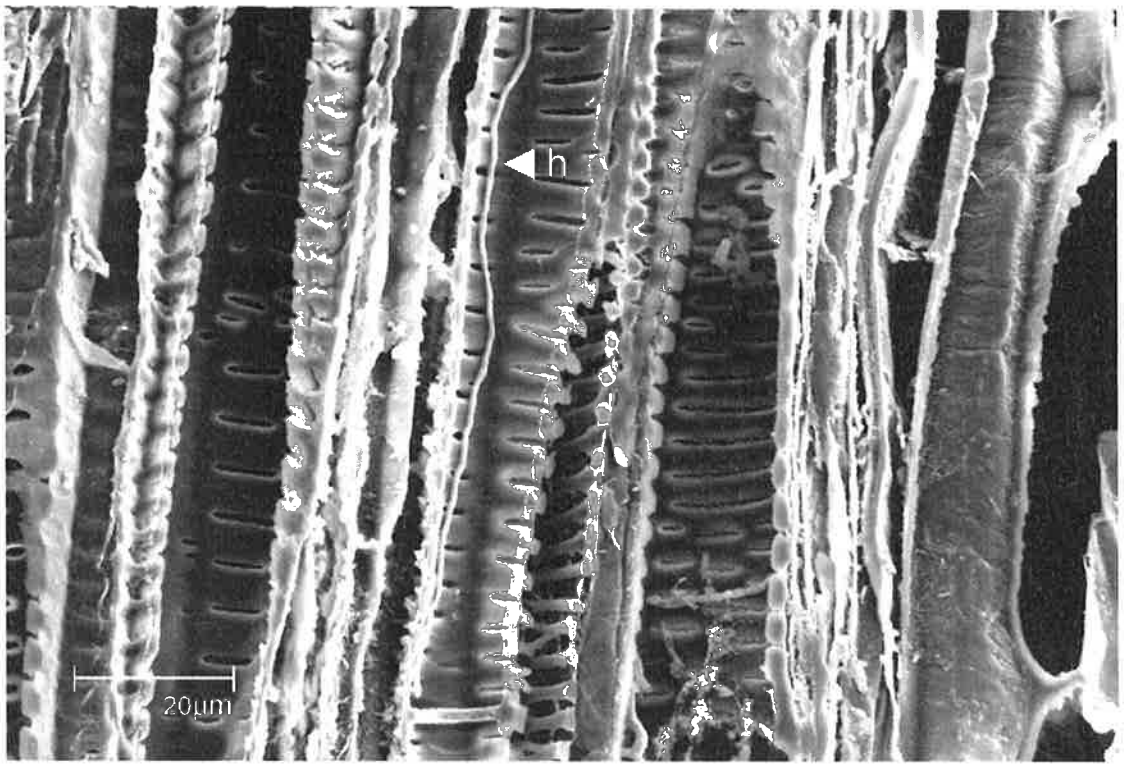


Figure 4.10: Scanning electron micrograph of a longitudinal section of the petiole of the blackleg resistant canola cv. Scoop, 28 days after inoculation with A-group *L. maculans* isolate 66/97. A hypha (h) extends through the xylem vessel from the leaf towards the stem.

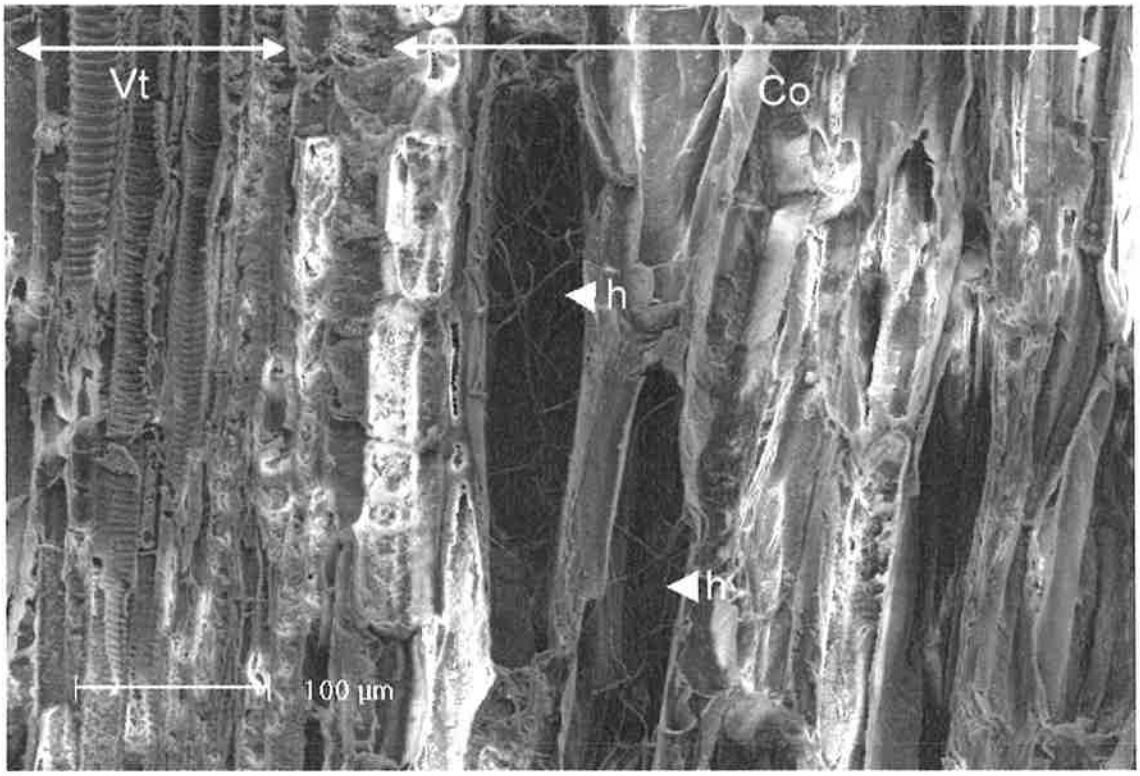


Figure 4.11: Scanning electron micrograph of a longitudinal section of the petiole of the blackleg resistant canola cv. Scoop, 28 days after inoculation with A-group *L, maculans* isolate 66/97. Hyphae (h) have colonised cells in the cortex (Co) next to the vascular tissue (Vt).

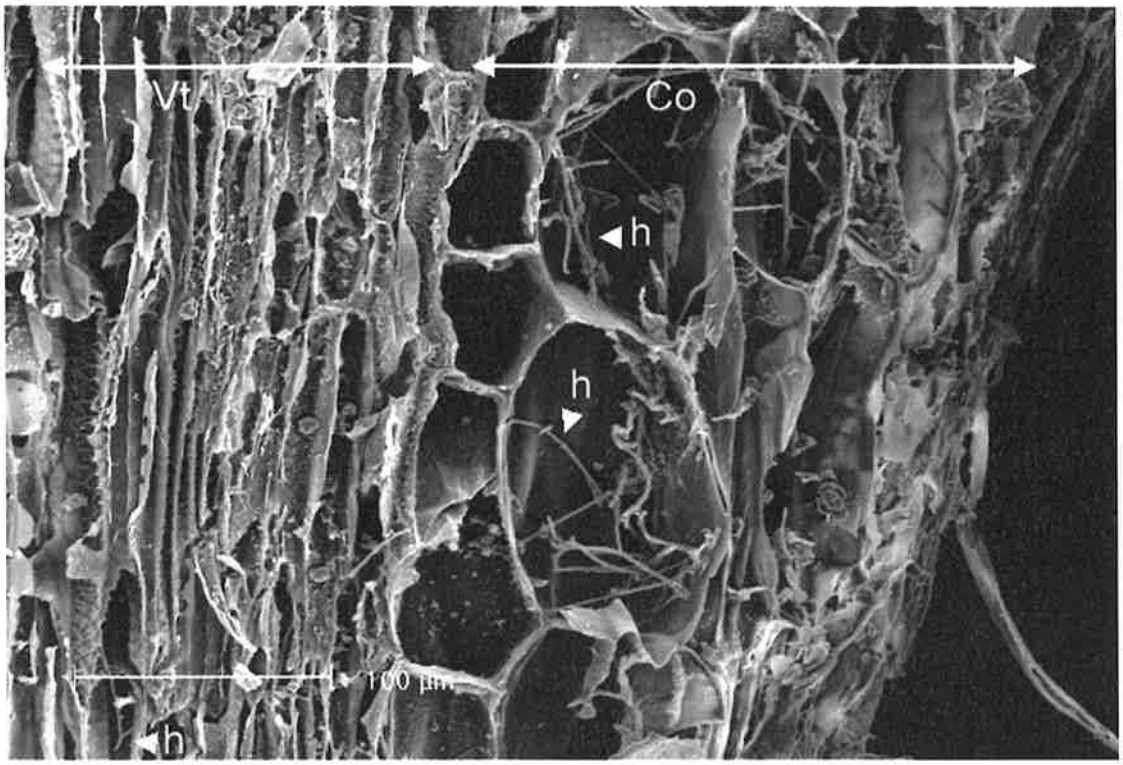


Figure 4.12: Scanning electron micrograph of a longitudinal section of the stem of the blackleg susceptible cv. Westar, 23 days after inoculation with the A-group *L. maculans* isolate 66/97. Hyphae (h) occur in the xylem vessel of vascular tissue (Vt) and the cells of the cortex (Co).

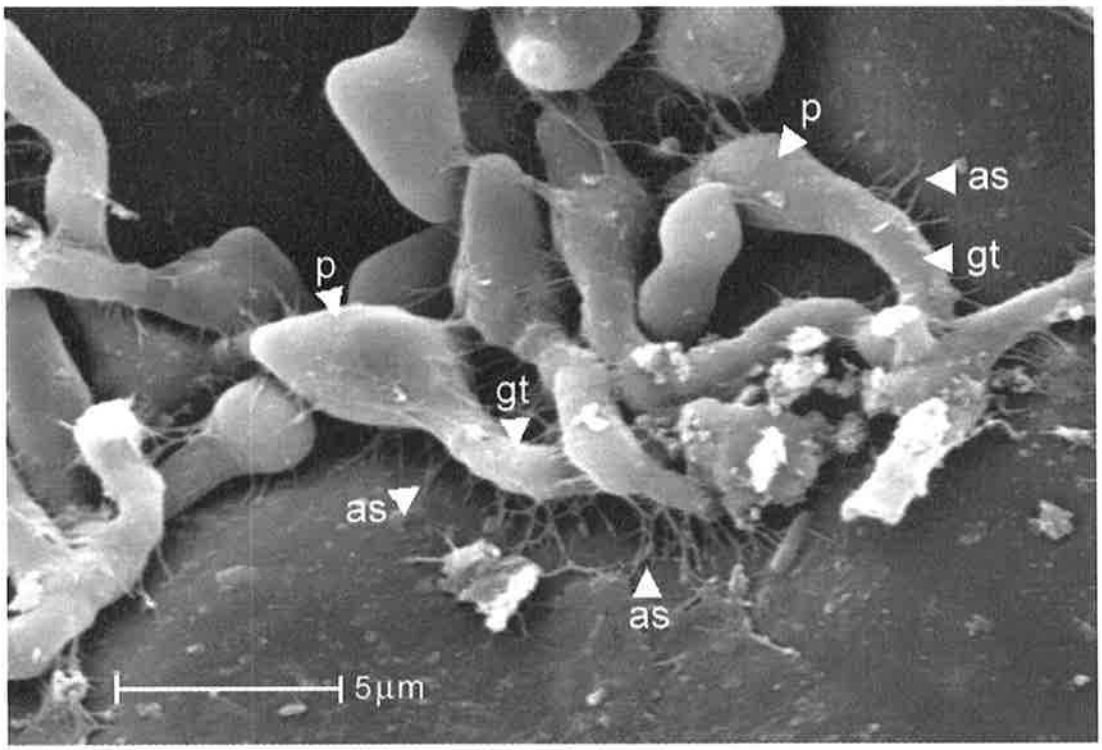


Figure 4.13: Scanning electron micrograph of *L. maculans* pycnidiospores on the adaxial leaf surface of the blackleg susceptible canola cv. Q2, 7 days after inoculation with A-group isolate 66/97. Pycnidiospores (p) and germ tubes (gt) appear to be attached to the epidermis with an adhesive substance (as).

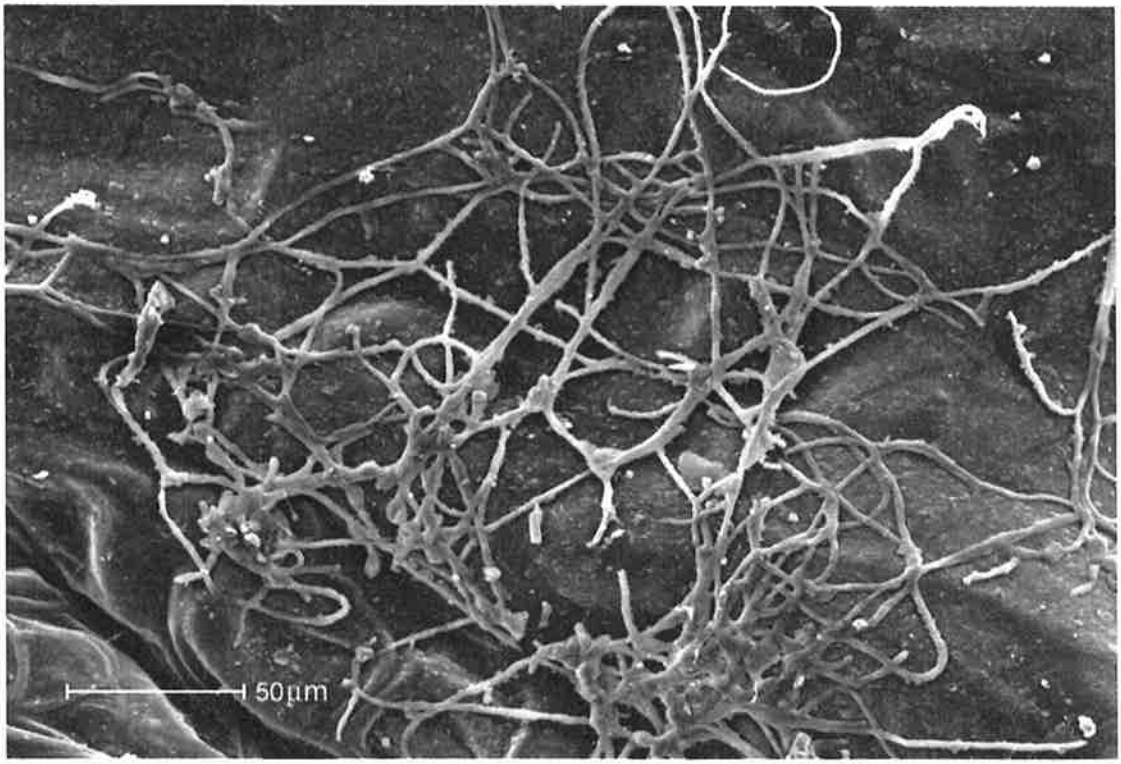


Figure 4.14: Scanning electron micrograph of an adaxial leaf surface of the blackleg susceptible cv. Q2, 14 days after inoculation with a pycnidiospore suspension of the A-group *L. maculans* isolate 66/97. Hyphae formed a mycelial mat over the surface of the leaf.

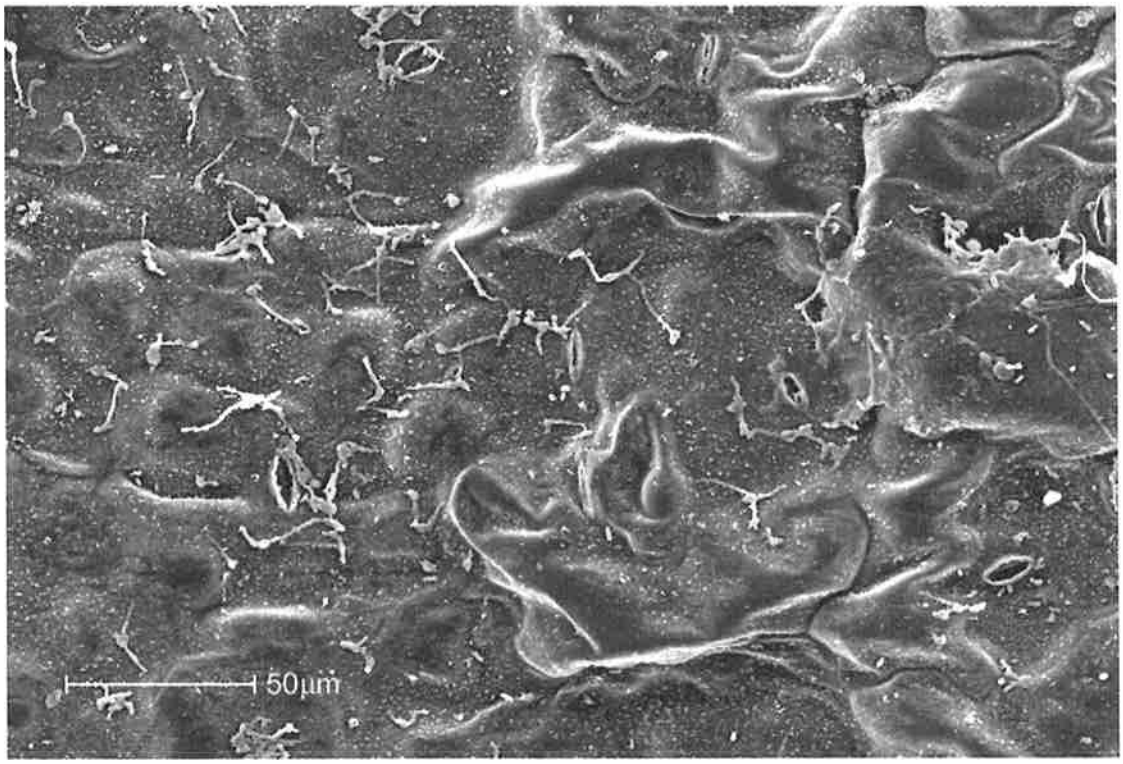


Figure 4.15: Scanning electron micrograph of an adaxial leaf surface of the blackleg resistant cv. Hyola 60, 14 days after inoculation with a pycnidiospore suspension of the A-group *L. maculans* isolate 66/97. Pycnidiospore germination was sparse and hyphal growth appeared restricted.

4.3.2 Histochemistry

4.3.2.1 Leaves

Microscopic examination of leaves of the resistant cv. Hyola 60, which were treated with phloroglucinol-HCl, revealed pronounced red-brown staining in the walls of epidermal cells surrounding stomata of plants which had been inoculated with *L. maculans* isolate 66/97 (Figure 4.16A). No such staining was observed in the cell walls of leaves from control plants (Figure 4.16B). Similarly, leaves of Hyola 60 that were treated with toluidine blue O had pronounced blue-green staining in walls of cells associated with stomata of inoculated plants (Figure 4.16C) and no staining was observed in the cell walls of control plants (Figure 4.16D). Staining of cell walls occurred consistently in inoculated leaves regardless of whether or not hyphal penetration had occurred, and not all stomata had cells with stained walls surrounding them. There was no staining of the stomatal guard cells.

Pronounced red-brown staining was observed in the walls of epidermal cells surrounding some stomata of all leaves of the resistant cv. Pinnacle (Figure 4.17A) which were inoculated with *L. maculans* isolate 66/97 and treated with phloroglucinol-HCl. Staining also consistently occurred in walls of cells surrounding some stomata on inoculated leaves of the susceptible cv. Q2 treated with phloroglucinol-HCl (Figure 4.17B). Inoculated leaves of cv. Pinnacle treated with toluidine blue O consistently revealed pronounced blue-green staining in the walls of epidermal cells surrounding some stomata (Figure 4.17C). The walls of cells surrounding stomata in leaves of the susceptible cv. Q2 were not stained when treated with toluidine blue, even when stomata had been penetrated by hyphae (Figure 4.17D). Red-brown staining occurred in the walls of cells surrounding some stomata in all the inoculated leaves of cvs. Pinnacle and Hyola 60 which were treated with Sudan IV (Figure 4.17E). Cv. Q2 also had some staining of the walls of cells associated with stomata of inoculated leaves treated with Sudan IV (Figure 4.17F), however the staining was not as intense or as frequent

as in cv. Pinnacle. Stomatal guard cells of cvs Pinnacle and Q2 were also stained when treatments were applied. Staining was not observed in either cultivar when uninoculated leaves were treated with any of the stains.

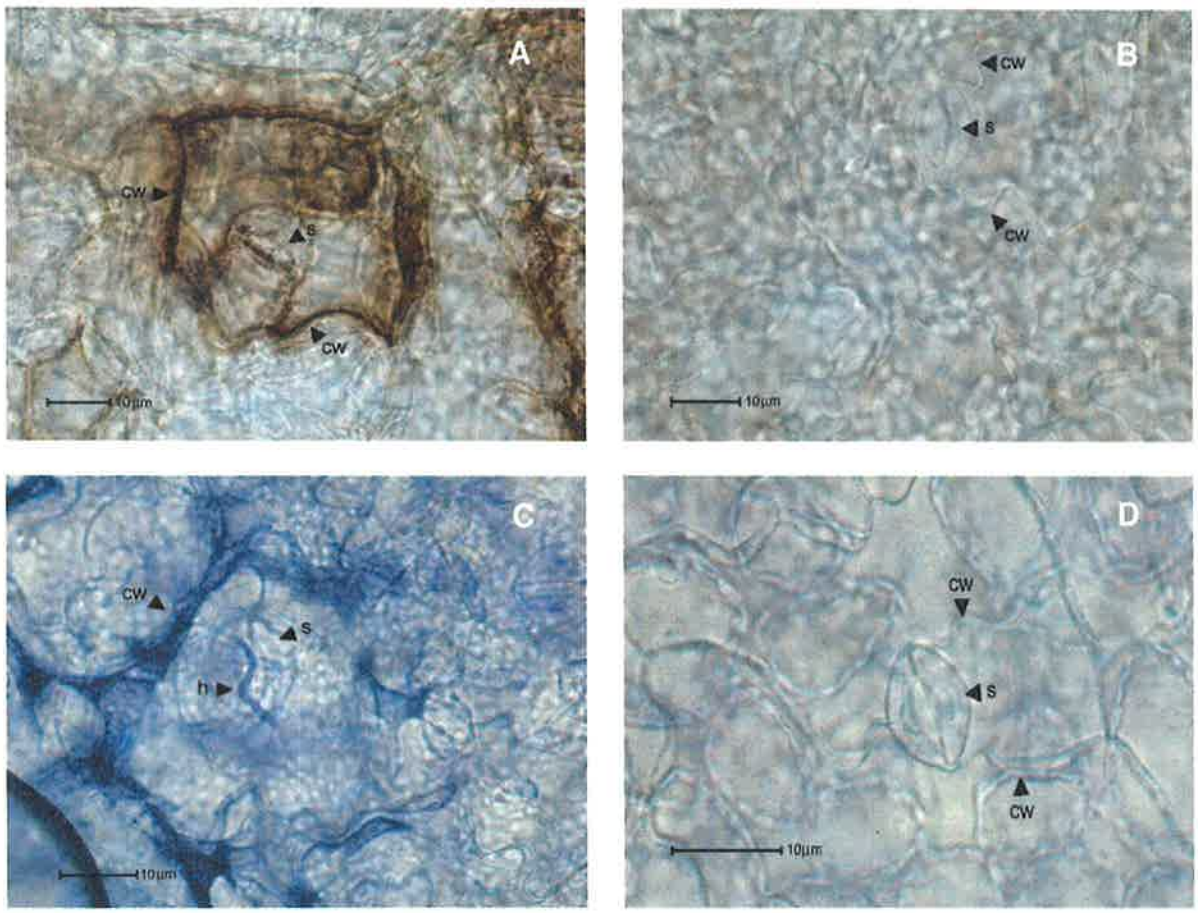


Figure 4.16: Light microscopic images of the adaxial leaf surface of blackleg resistant canola plants (cv. Hyola 60), treated with histochemical stains 11 days after inoculation with *L. maculans* isolate 66/97 and uninoculated controls. A. Inoculated leaves with red-brown staining by phloroglucinol-HCl in walls of cells (cw) surrounding the stoma (s). B. Control leaves showing cell walls (cw) and stoma (s) which did not react with phloroglucinol-HCl. C. Inoculated leaves with blue staining by toluidine blue O in the walls of cells (cw) surrounding a stoma (S) which had been penetrated by a hypha (h). D. Control leaves showing cell walls (cw) and a stoma (s) which did not react with toluidine blue O.

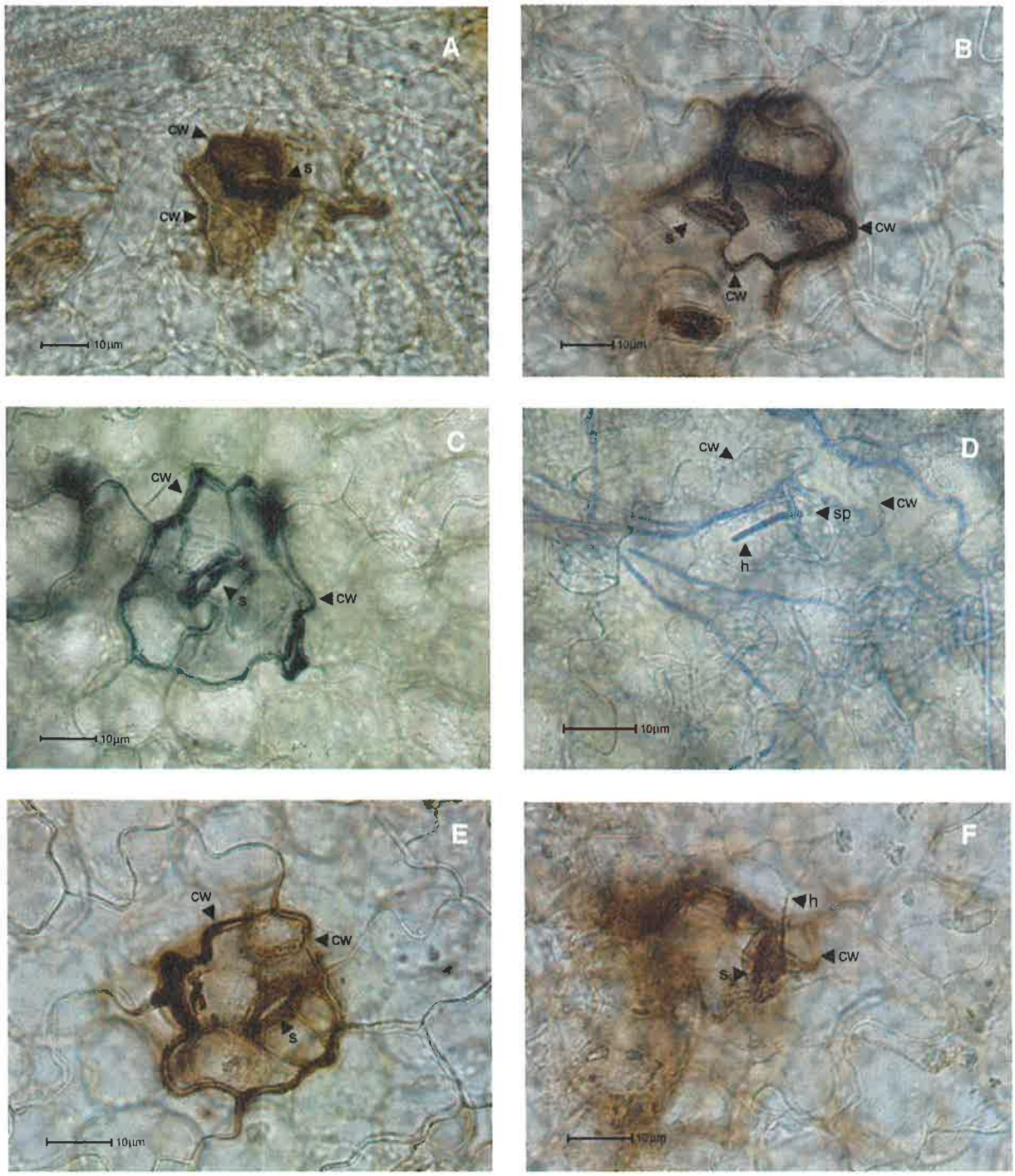


Figure 4.17: Light microscopic images of the adaxial leaf surface of canola plants, treated with histochemical stains 11 days after inoculation with the *L. maculans* isolate 66/97 and uninoculated controls. A. Inoculated blackleg resistant cv. Pinnacle with red-brown staining by phloroglucinol-HCl in the walls of cells (cw) surrounding the stoma (s), including guard cells. B. Inoculated blackleg susceptible cv. Q2 with red-brown staining by phloroglucinol-HCl in the walls of cells (cw) surrounding the stoma (s), including guard cells. C. Inoculated cv. Pinnacle with blue-green staining by toluidine blue O in the walls of cells (cw) surrounding the stoma (s), including guard cells. D. Inoculated cv. Q2 with no staining by toluidine blue O in the walls of cells (cw) surrounding a stoma which has been penetrated (sp) by a hypha (h). E. Inoculated cv. Pinnacle with red-brown staining by Sudan IV in the walls of cells (cw) surrounding the stoma (s), including guard cells. F. Inoculated cv. Q2 with red-brown staining by Sudan IV in the walls of cells (cw) surrounding a stoma (s) which has been penetrated by a hypha (h).

4.3.2.2 Stems

The anatomy of the canola stem is illustrated in Figure 4.18. In the centre of the stem large cells make up the pith. The pith is surrounded by a vascular cylinder, which comprises the xylem on the inner edge, phloem on the outer edge and a thin band of cambium separating them. The cortex surrounds the vascular cylinder and the epidermis forms the outermost layer of cells of the stem.

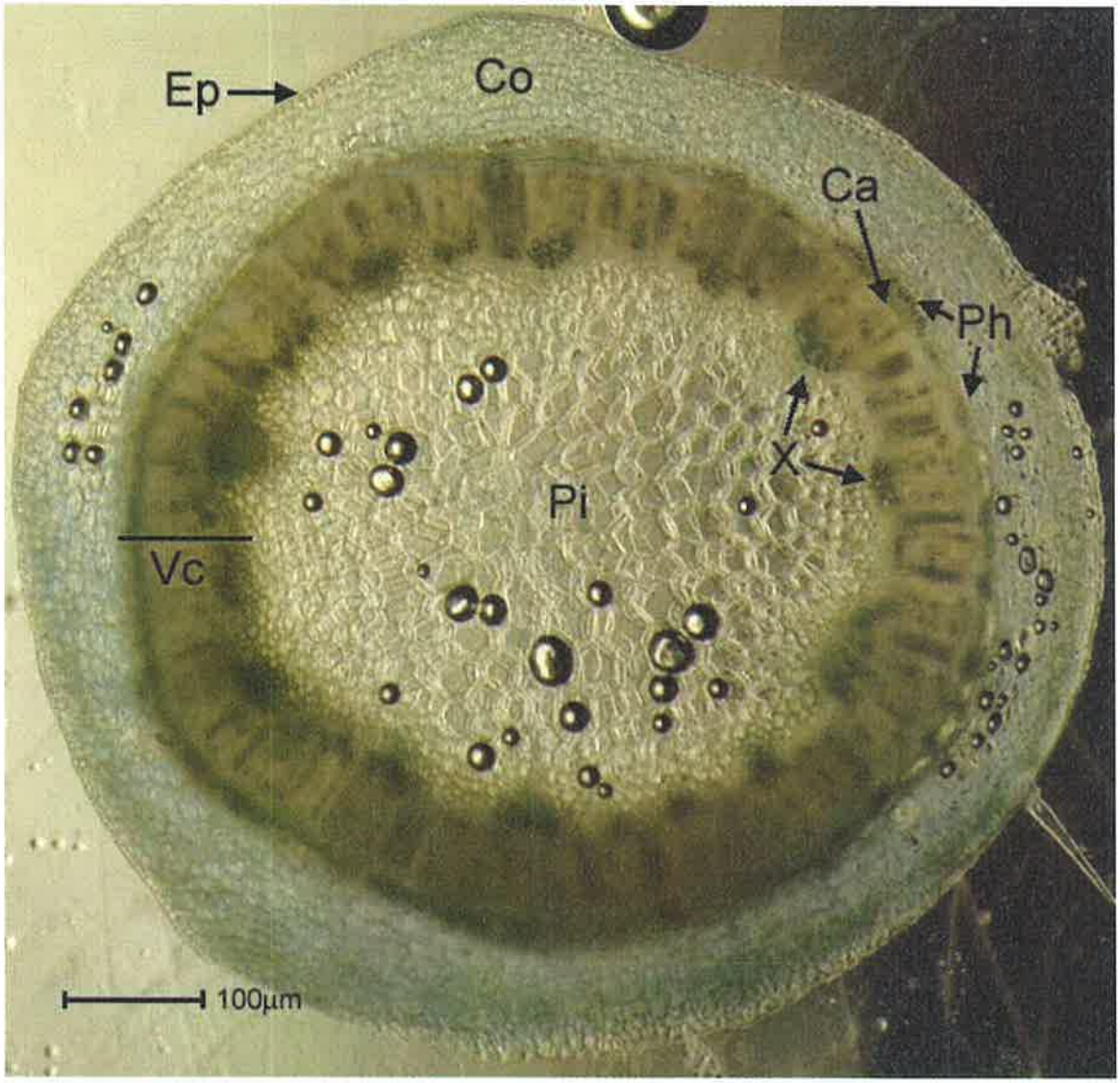


Figure 4.18: Light microscopic image of the cross-sectional view of a canola stem (cv. Hyola 60) after staining with aniline blue. It illustrates the structure of the stem with the pith (Pi) surrounded by a vascular cylinder (Vc) which is in turn surrounded by the cortex (Co) and finally the epidermis (Ep). Within the vascular cylinder are the xylem (X) and phloem (Ph) regions wich contain xylem vessels and sieve tubes respectively, and are separated by a narrow band of cambium (Ca). Anatomical structures were identified using Salisbury and Ross (1992) and Curtis and Barnes (1989).

When inoculated plants of cv. Hyola 60 were treated with phloroglucinol-HCl, the xylem and phloem regions were stained a purple-red colour, with more intense staining around the larger xylem vessels (Figure 4.19A). The larger xylem vessels of cvs Q2 and Pinnacle were stained red-brown (Figure 4.19B) after stem sections were treated with phloroglucinol-HCl, but all other structures were unstained. When sections of stem of cvs Hyola 60, Pinnacle and Q2 were treated with toluidine blue O, the xylem and phloem regions were stained blue-green and the xylem vessels and sieve tubes were stained dark blue (Figure 4.19C&D).

Stem sections of cvs Hyola 60, Pinnacle and Q2 which were treated with aniline blue had dark blue-green staining of the large xylem vessels, but the sieve tubes were unstained (Figure 4.19E&F). Treatment of stem sections with Sudan IV produced red-brown staining around the xylem vessels of cvs Hyola 60, Pinnacle and Q2, and sieve tubes appeared unstained (Figure 4.19A&B).

Control plants did not differ from the inoculated plants for all treatments. This is illustrated by treatment of the uninoculated cv. Q2 with toluidine blue O (Figure 4.20C) and Sudan IV (Figure 4.20D), which produced blue-green and red-brown staining, respectively, of the xylem vessels, and only blue-green staining of the sieve tubes.

Figure 4.20E shows the orange-brown staining of xylem vessels with Sudan IV and Figure 4.20F shows the blue staining of sieve tubes with toluidine blue O, at higher magnification in stem sections of the inoculated cv. Hyola 60.

A summary of all the staining patterns observed in leaves and stems of treated canola plants is given in Table 4.1.

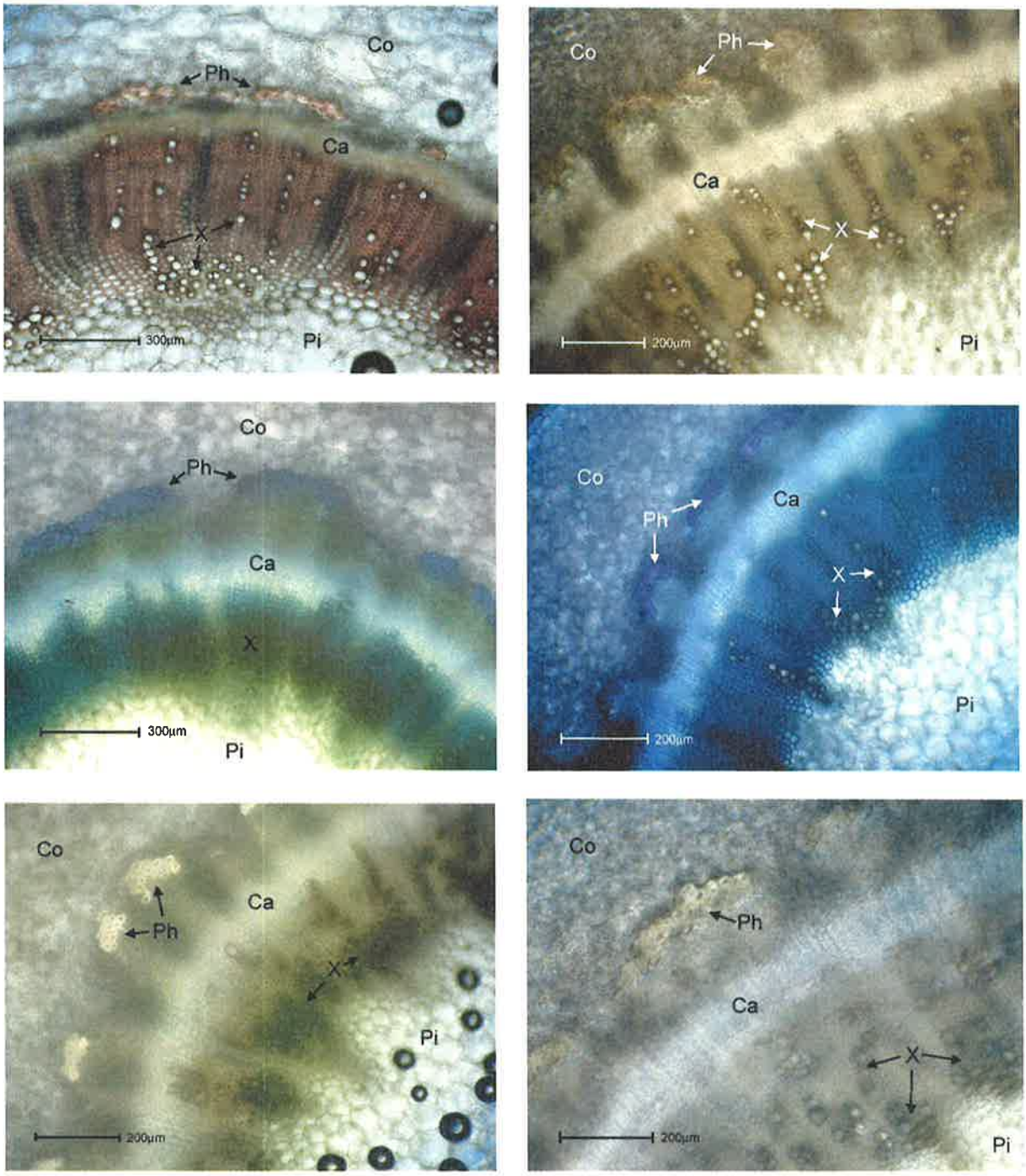


Figure 4.19: Light microscopic images of the cross-sectional view of stems from canola plants 28 days after inoculation, via the leaves, with *L. maculans* isolate 66/97. Each image shows a section of the vascular cylinder which comprises the xylem (X) and phloem (Ph) regions, separated by the cambium (Ca), and flanked by the pith (Pi) and the cortex (Co) regions. A. Xylem vessels and sieve tubes (phloem) in the resistant cv. Hyola 60, treated with phloroglucinol-HCl, were stained purple-red. B. Xylem vessels in the susceptible cv. Q2 treated with phloroglucinol-HCl, were stained red-brown. C. Xylem vessels and sieve tubes in the resistant cv. Pinnacle treated with toluidine O were stained blue-green. D. Xylem vessels and sieve tubes in cv. Q2 treated with toluidine O were stained blue-green. E. Xylem vessels in cv. Pinnacle treated with aniline blue were stained blue-green. F. Xylem vessels in cv. Q2 treated with aniline blue were stained blue-green.

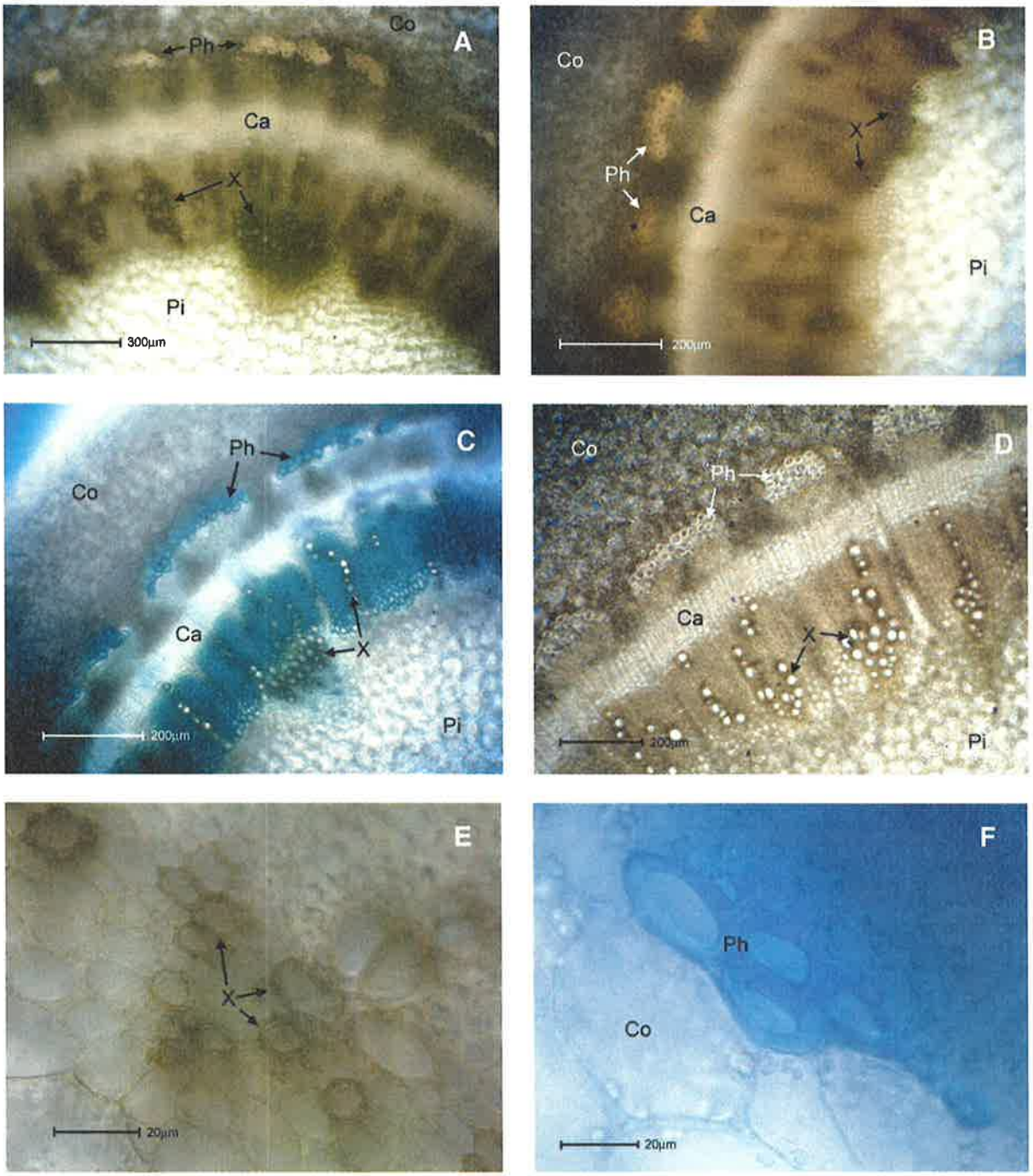


Figure 4.20: Light microscopic images of the cross-sectional view of stems from canola plants 28 days after inoculation (images A, B, E & F), via the leaves, with the A-group *L. maculans* isolate 66/97. Stems in images C & D were from control plants, subjected to RO water on the leaves only. Images A - D show a section of the vascular cylinder which comprises the xylem (X) and phloem (Ph) regions, separated by the cambium (Ca), and flanked by the pith (Pi) and the cortex (Co) regions. A. Xylem vessels of the resistant cv. Pinnacle treated with Sudan IV were stained red-brown. B. Xylem vessels of the susceptible cv. Q2 treated with Sudan IV were stained red-brown. C. Xylem vessels and sieve tubes (phloem) of cv. Q2 (control) treated with toluidine blue O were stained blue-green. D. Xylem vessels of cv. Q2 (control) treated with Sudan IV were stained red-brown. E. Xylem vessels (X) of cv. Hyola 60 treated with Sudan IV were stained red-brown. F. Sieve tubes in the phloem region (Ph) of the resistant cv. Hyola 60 treated with toluidine blue O were stained blue, and adjacent cells of the cortex (Co) were unstained.

Table 4.1: Responses by leaves and stems of canola cvs Hyola 60 (highly blackleg resistant), Pinnacle (resistant) and Q2 (susceptible) to treatments which detect the presence of lignin (phloroglucinol-HCl, toluidine blue O), suberin (Sudan IV) and callose (aniline blue). Plants were either inoculated with the *L. maculans* isolate 66/97 as droplets of pycnidiospore suspension or with RO water only (control).

Leaves		Phloroglucinol-HCl	Toluidine blue O	Sudan IV	Analine blue
Hyola 60	inoculated	red-brown (ECW)	blue-green (ECW)	red-brown (ECW)	na
	control	no response	no response	no response	na
Pinnacle	inoculated	red-brown (ECW, GC)	blue-green (ECW, GC)	red-brown (ECW, GC)	na
	control	no response	no response	no response	na
Q2	inoculated	red-brown (ECW, GC)	no response	red-brown (ECW, GC)	na
	control	no response	no response	no response	na
Stems					
Hyola 60	inoculated	purple-red (XV, ST)	blue-green (XV, ST)	red-brown (XV)	blue-green (XV)
	control	purple-red (XV, ST)	blue-green (XV, ST)	red-brown (XV)	blue-green (XV)
Pinnacle	inoculated	red-brown (XV)	blue-green (XV, ST)	red-brown (XV)	blue-green (XV)
	control	red-brown (XV)	blue-green (XV, ST)	red-brown (XV)	blue-green (XV)
Q2	inoculated	red-brown (XV)	blue-green (XV, ST)	red-brown (XV)	blue-green (XV)
	control	red-brown (XV)	blue-green (XV, ST)	red-brown (XV)	blue-green (XV)

ECW – epidermal cell wall, GC – guard cell, XV – xylem vessel, ST – sieve tube, na – not available

4.3.3 Progress through the petiole

The distance over which hyphae of *L. maculans* extended ahead of the leading edge of the lesion on the petiole was variable for both the susceptible cv. Hyola 42 and the resistant cv. Dunkeld. Of nine petioles collected from Hyola 42 plants, one yielded no cultures of *L. maculans* at all, five yielded *L. maculans* a maximum of 2-14 mm from the lesion and three yielded *L. maculans* a maximum of 36-44 mm from the lesion (Table 4.2). In three petioles from Hyola 42, *L. maculans* could not be isolated from some sections although it was isolated from sections further away from the lesion. Of the seven petioles collected from Dunkeld plants, five yielded *L. maculans* a maximum of 2-8 mm from the lesion, one yielded *L. maculans* a maximum 12 mm from the lesion and one yielded *L. maculans* a maximum of 18 mm from the lesion (Table 4.2). *L. maculans* could not be isolated from sections in two

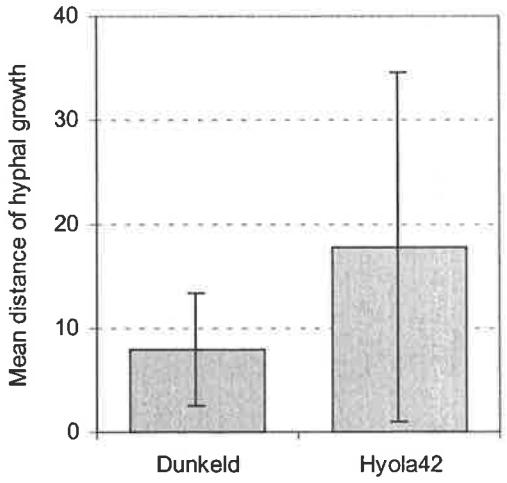
petioles from cv. Dunkeld, although it was isolated from sections further away from the lesion.

Table 4.2: Sequential sections (2 mm each) of petioles beyond the margin of the lesion. Max is the maximum distance *L. maculans* was isolated from the margin of the lesion. + denotes positive identification of *L. maculans*, - denotes no *L. maculans* present.

Cultivar	Petiole	Distance (mm)																						Max
		2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34	36	38	40	42	44	
Hyola 42	1	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14
	2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	36
	3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
	4	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	10
	5	+	+	+	+	+	+	+	+	+	-	+	-	-	-	-	-	+	+	+	-	-	-	38
	6	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
	7	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8
	8	+	+	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	-	+	-	+	+	44
	9	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6
Dunkeld	1	+	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	12
	2	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6
	3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
	4	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	4
	5	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	8
	6	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6
	7	+	+	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	18

A one-way analysis of variance, where the dependent variable was maximum distance from the lesion that *L. maculans* was isolated in the petiole, revealed there was no significant difference ($P>0.05$) between cv. Hyola 42 and cv. Dunkeld. The mean distance *L. maculans* was isolated along the petiole from the lesion was 17.7 mm in cv. Hyola 42 and 8 mm in cv. Dunkeld (Figure 4.21). Two collecting dates were used to gather sufficient samples for analysis and the effect of the two dates on progress of hyphae through the petiole was not analysed.

Figure 4.21: Mean distance of hyphal growth through the petiole from the leading edge of the necrotic lesion. Bars = standard deviation



4.4 DISCUSSION

Pycnidiospores, which land on the leaf surface of canola plants, must attach themselves to the cuticle to prevent dislodgement due to wind or rain splash. It appears that they may do this by means of an adhesive substance which anchors them to the waxy cuticle. However, this was observed only in a few instances, and further work is necessary to discount the possibility that the "stringy substance" is an artefact of specimen preparation. It is possible that the dehydration steps may have washed the substance away on other leaves, and this could be overcome by using an environmental scanning electron microscope (ESEM), which allows examination of fresh samples. Some evidence exists for the presence of adhesive compounds in other plant-fungus interactions. Shaw and Hoch (1999) reported the secretion of a proteinaceous material by pycnidiospores of *Phyllosticta ampelicida*, which allowed attachment to grape leaves. Mucilage was responsible for attachment of conidia of *Fusarium solani* var. *phaseoli* to roots of *Vigna radiata* (Schuerger and Mitchell 1993).

The timing of pycnidiospore germination and germ tube growth on the leaf surface of canola plants appeared to be variable and there was no obvious difference between resistant and susceptible cultivars. Sosnowski *et al.* (2001b) showed that there were no significant

differences between cultivars with respect to germination percentage of pycnidiospores. As hyphae grew randomly across the surface of the leaf, only some stomata were penetrated, confirming observations by Sosnowski *et al.* (2001b). In this study, hyphae penetrated the leaf via stomata and wounds, as was reported by Hammond and Lewis (1987a) and Chen and Howlett (1996) also. Scanning electron micrographs revealed that hyphae might produce a compound, which causes the waxy cuticle and epidermis to contract and tear open. This would allow pathogen entry into the leaf, facilitating greater colonistation of the leaves. However, further work is necessary to discount that the tearing is an artefact of specimen preparation.

The cv. Hyola 60, which has very high stem resistance to *L. maculans* according to national cultivar trials (Potter and Stanley 2002), was shown to have significantly fewer leaf and cotyledon lesions in field trials than all other cultivars tested (section 5.3.1). SEM images of the surface of leaves 14 days after inoculation revealed that pycnidiospore germination was inhibited and hyphal growth severely restricted on cv. Hyola 60 compared to the susceptible cv. Q2. It is likely that the reduction of leaf infection would decrease the number of hyphae that reach the stem. Therefore the mechanisms of resistance in the stem would be more effective than in other cultivars where risk of disease is greater. It is possible that Hyola 60 produces an anti-fungal biochemical compound in the leaves, which does not occur in unrelated cultivars. The resistance in Hyola 60 originates from a wild accession of *B. rapa* var. *sylvestris* which was collected in Sicily and then crossed with *B. oleracea* var. *albogabra* to produce a hybrid (Crouch *et al.* 1994).

The walls of epidermal cells surrounding stomata in leaves of Hyola 60 and Pinnacle were stained with phloroglucinol-HCl and toluidine blue O, only in leaves infected by *L. maculans*. This showed that lignification of the epidermal cells occurred in response to infection.

However, in the susceptible cv. Q2, cell walls stained in the presence of phloroglucinol-HCl, but not toluidine blue O. There are several possible reasons for this; 1) the concentration of lignin may be lower in the susceptible cv. Q2 than in the resistant cvs Hyola 60 and Pinnacle, 2) toluidine blue O could be less sensitive to lignin than phloroglucinol-HCl and 3) toluidine blue O may be reacting with a compound other than lignin. Treatment with Sudan IV revealed that suberin was also produced in the walls of epidermal cells and guard cells surrounding stomata in response to infection. Both lignin and suberin appeared to be more diffuse in the epidermal cell walls of the susceptible cv. Q2, which suggested that lower concentrations were present than in the resistant cultivars. Lignin and suberin accumulation in cell walls occurred whether stomata had been penetrated by hyphae or not, therefore recognition of infection must occur before hyphae reach the stomata. Chen and Seguin-Swartz (1999) detected lignification in tissue around *L. maculans* infection sites in several wild crucifers after treatment with phloroglucinol-HCl. Therefore, lignification and suberin accumulation of cell walls are two mechanisms by which *B. napus* resists infection by *L. maculans*, in addition to several other methods (Xi and Morrall 1993; Roussel *et al.* 1999a; Sosnowski *et al.* 2001b), however it does not appear to be the main mechanism of resistance in current Australian cultivars.

Scanning electron micrographs revealed that hyphae grew along the xylem vessels of petioles and colonised the cortical cells in both resistant and susceptible cultivars. However, hyphae were first observed in the susceptible and resistant cultivars, 13 and 17 days after inoculation respectively, using the SEM. The distance which hyphae were isolated from the margin of the petiole lesion was not significantly different between the resistant and susceptible cultivars due to the large variation in results. However, hyphae extended up to 44 mm in front of the lesion in the susceptible cultivar and only 18 mm in the resistant cultivar. Therefore, it is possible that hyphae have greater potential to extend at a faster rate through

the petiole of susceptible plants, suggesting that growth rate may be restricted in the petiole of the resistant plant.

Hyphae were observed to have grown in the xylem vessels of the stem, but could not be seen in the stem/petiole junction. Cutting sections of the stem/petiole junction with a razor blade without destroying the integrity of the structure was difficult and may explain why hyphae could not be seen. Hammond *et al.* (1985) observed that once the hyphae of *L. maculans* reached the stem of *B. napus* plants, they began to colonise the cortical cells immediately. In this study, hyphae were observed in the xylem vessels and cortex of stems of susceptible plants but not resistant plants. This suggested that resistant cultivars restrict hyphae of *L. maculans* from growing out of the petiole and into the stem.

Histochemical stains were applied to stem sections from both resistant and susceptible cultivars to detect the presence of lignin, suberin and callose. All of these compounds were detected, using light microscopy, in xylem vessels of both resistant and susceptible cultivars, and only lignin was present in sieve tubes. All three compounds were observed in the control plants, hence, these compounds are not likely to be responsible for the resistance of stems to canker in current Australian cultivars, but may play a role in slowing the growth of the fungus. Hammond and Lewis (1987b) showed that lignification of xylem vessels in the stem along with the presence of callose, was associated with stem resistance of European cultivars of *B. napus* to *L. maculans* infection. Chen and Seguin-Swartz (1999) also found that lignification was responsible for preventing stem canker in stem tissues of some wild crucifer species in Canada.

Several techniques were used to examine the interactions between *L. maculans* and canola in leaves, petioles and stems. A number of possible resistance mechanisms were identified,

however, the mechanisms by which canola resists stem canker are still not completely understood. Resistance in the leaves has been somewhat overlooked by breeders in more recent times as a mechanism of preventing infection by *L. maculans*. The mechanism by which the cv. Hyola 60 restricts the fungus on the leaf surface appears to be very efficient and leads to a plant which is almost 'immune' to stem canker. Misaghi (1982) emphasised that resistance is usually the function of a number of mechanisms operating in an integrated, coordinated manner. Therefore, breeders could improve blackleg resistance in canola by identifying germplasm with leaf resistance and incorporating it into current cultivars.

CHAPTER 5

EPIDEMIOLOGY IN THE FIELD

5.1 INTRODUCTION

The maturation of pseudothecia on canola stubble is dependent on both temperature and moisture (Petrie 1994; Pérès *et al.* 1999). Warm or cold, dry conditions will delay pseudothecial maturation, whereas optimal temperatures of 14-15°C and moisture will ensure that maturation occurs by seedling emergence in autumn. However, too much moisture will encourage rapid decomposition of stubble, reducing its ability to produce inoculum (McGee 1977; Petrie 1978; West *et al.* 2001).

Rainfall or dew triggers ascospore release once the pseudothecia have matured (Barbetti 1975; McGee 1977; Williams 1992). The liberated ascospores can be carried up to 5 km by wind to land on the cotyledons and leaves of canola seedlings, then at least 4 h of continuous wetness and temperatures above 20°C are required for spore germination to occur (Hall 1992). Symptoms develop on cotyledons and leaves within 1 week of infection when temperatures remain between 15-20°C and leaf wetness duration is at least 48 h (Biddulph *et al.* 1999; Toscano-Underwood *et al.* 2001). Leaf lesions produce abundant pycnidia when the day/night temperature ranges between 18/11°C and 24/15°C (Barbetti 1975) and when pycnidiospres are produced, they require rain splash and wind to spread to other parts of the plant and neighbouring plants. Long periods of high humidity increase the rate of infection by pycnidiospores (McGee 1977; Xi *et al.* 1991).

L. maculans spreads from the leaves of infected plants into the stem, via the petioles. At the base of the stem, a canker can develop under optimal temperatures (20-24°C) and infection

will be restricted at low or high temperatures (Barbetti 1975: McGee and Petrie 1979; Gladders and Musa 1980).

An epidemic of blackleg is determined by many factors, most importantly, weather conditions. Resistance in some cultivars to blackleg will alter the effect that weather has on disease progress. In this chapter, the response of a range of canola cultivars to blackleg infection in the field under variable environmental conditions, is examined in detail. The aim was to improve our understanding of the epidemiology of blackleg and develop a model to forecast the disease and its effect on yield, which will, in turn, assist farmers with management of the disease.

5.2 MATERIALS AND METHODS

5.2.1 Field trials

Two field trials were sown in each of the 2000 and 2001 seasons. One trial was located at the Kingsford Research Station, which is approximately 7 km north of Gawler, South Australia. The second trial was located at the Charlick Research Station, approximately 9 km south of Strathalbyn, South Australia.

In 2000, both trials were set up in the same manner at the two locations and sown on May 10 and 11 for the Charlick and Kingsford trials, respectively. Eight canola cvs; Dunkeld, Mystic, Scoop, Oscar, Pinnacle, Monty, Karoo, Hyola 42 and Q2, were selected to represent a range of resistance to blackleg and maturity (Table 3.1). Plots were 10 m x 8 rows with 17.8 cm spaces (Figure 5.1A) and each one was sown with one variety at 5 kg seed/ha, with 2 m pathways between rows for access by spraying vehicles. The trial was a randomised block design with three replications and buffer plots (cv. Monty) were sown around the border of the trial (Appendix 5, Figure 5.1B). In 2001, both trials were set up as in 2000, with the

addition of cv. Hyola 60, and sown on May 25 and 31 for the Charlick and Kingsford trials respectively. Trial sites were prepared by the farm managers at each of the research stations and sown, maintained and harvested by the SARDI Field Crop Evaluation Group. Information on the fields used for the trials is given in Appendix 6.

5.2.1.1 Barley inoculum

To promote spread of disease in the trials, barley grain colonised with *L. maculans* was prepared, based on methods used by Roger and Tivoli (1996) for inoculation of field peas with *Mycosphaerella pinodes*. Barley grain (150-200 g) was placed into oven bags (Glad, 35 x 48 cm), with enough tap water to cover the grain, then left to soak overnight. Excess water was poured off and then the bags were closed with sterile cotton plugs wrapped in muslin. The bags were autoclaved for 60 min at 121°C, cooled to 22°C for 24 h and then placed into a second bag before being autoclaved for a second cycle (60 min at 121°C). Using aseptic techniques in a laminar flow unit, 5 ml of pycnidiospore suspension, prepared as in section 3.2.1.4, was added to each bag and shaken. Virulent isolates of *L. maculans* collected in previous years, were used; 66/97, 126/99 and 208/00 at Kingsford and 66/97, 127/99 and 165/98 at Charlick. Bags of grain were inoculated with a pycnidiospore suspension (10^6 pycnidiospores/ml RO water as in section 3.2.1.4) of each isolate separately and then incubated for 5 weeks on a bench in the laboratory (Figure 5.1C) at approximately 24°C under diurnal fluorescent light (Osram cool white 36W/21-840). Barley grains colonised with isolates of *L. maculans* (Figure 5.1D) were mixed for each site and evenly spread over the surface of the soil in each plot (Figure 5.1D) at approximately 20 g (dry wt.) per m^2 at both sites on June 15, 2000 and June 11, 2001, when plants were at GS 2.1 – 2.3.

5.2.1.2 Disease assessment

Once most cotyledons were fully developed, 10 plants in each plot were marked at random by inserting 600 x 6 mm dowels into the soil adjacent to the plants (Figure 5.1A). The top half of each dowel was painted fluorescent pink to make them easily identifiable when the plants grew large. Each marked plant was assessed weekly throughout the season for disease severity using the three rating scales (cotyledon, leaf and stem) given in Appendix 7. The weekly mean disease incidence was determined by counting the number of plants in each plot with any blackleg symptoms on either cotyledons, leaves or stems. The mean severity of leaf infection was not calculated for trials in 2001. The weekly mean stem canker incidence was determined by counting the number of plants in each plot with blackleg stem canker (i.e. stem rating 5 or greater). After the final rating, all trials except for Charlick 2000 were harvested and the seed from each plot was weighed to estimate yield (t/ha).

5.2.2 Spore trap plants

Two trays containing nine punnets (900 ml) each were filled with potting soil and sown with seed of cv. Hyola 42 (three seeds per punnet – thinned to two plants per punnet). Plants were grown in the glasshouse (18-29°C) and watered daily (Figure 5.1F). Once plants reached GS 2.3 – 2.4, one tray was placed at least 20 m from each trial. Plants were left exposed to air-borne inoculum for 1 week under a wire cage to prevent animals from feeding on them (Figure 5.1G). The trays were returned to the glasshouse, incubated for 4 days in plastic bags, then left on a bench for a further 6 days and watered. Plants were assessed 10 days after returning from the field by counting the number of leaf lesions caused by *L. maculans*. This routine was repeated each week throughout the 2000 and 2001 seasons from sowing until the field trials were harvested.

5.2.3 Weather data collection

Weather data were collected every 15 minutes and averaged over each day during the growing season using Automatic Weather Stations (AWS) manufactured by Measurement Engineering Australia Pty Ltd. Identical AWS systems were set up at each trial site (Figure 5.1H). They included sensors for the following parameters; air temperature, canopy temperature, soil temperature, air relative humidity, canopy relative humidity, rainfall and leaf wetness. Each AWS contained a data logger and information was downloaded using Magpie for Windows® software and converted into Microsoft Excel® for data management. Data for wind speed and direction as well as all the parameters outside of the time outside of the growing season were obtained from the Bureau of Meteorology, collected by AWS stations at Roseworthy (4 km north west of Kingsford RS) and Strathalbyn (9 km north of Charlick RS).

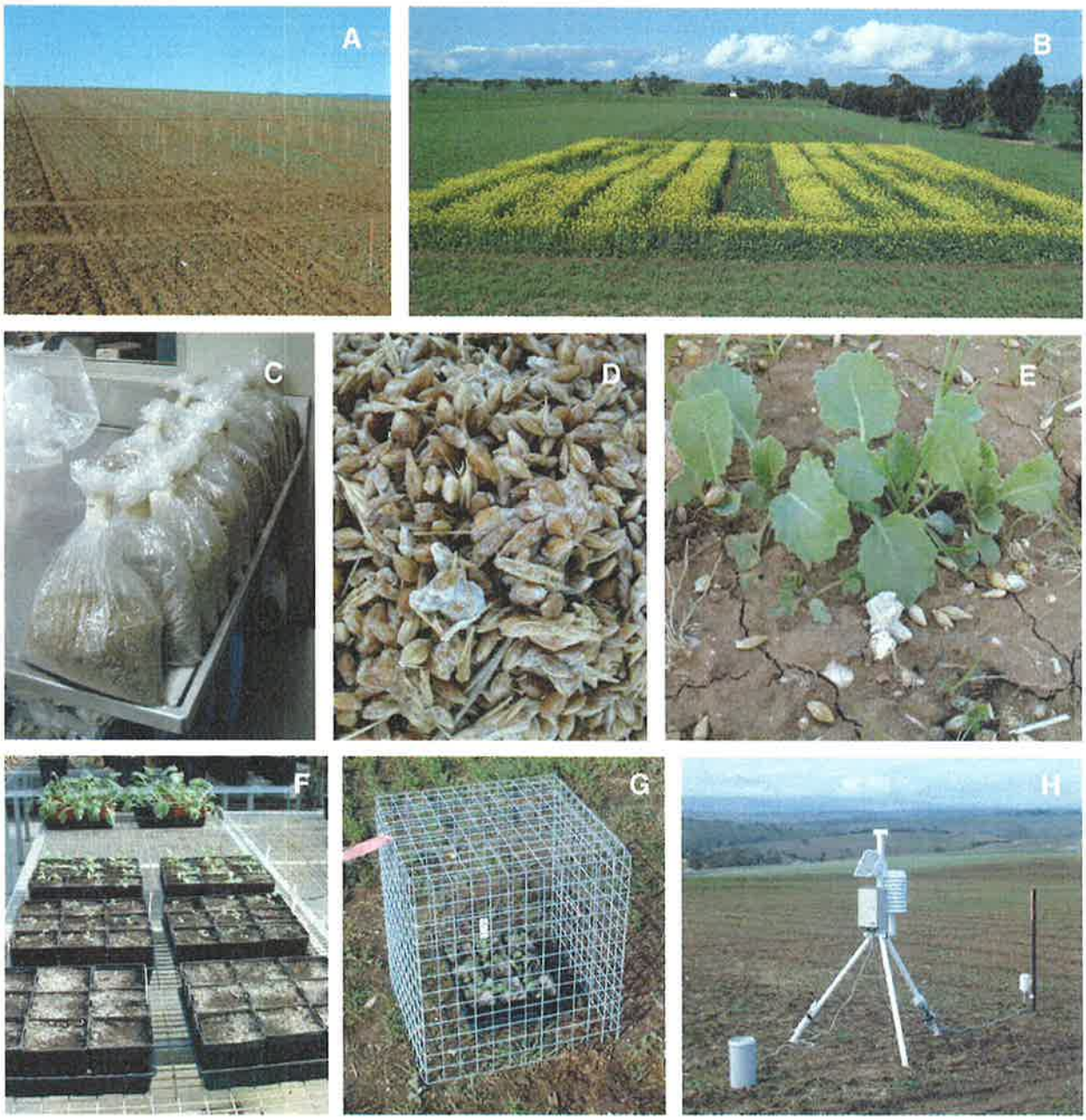


Figure 5.1: A. Field trial plots (10 m x 8 rows) with dowels which marked individual seedlings selected for assessment throughout the season. B. Charlick field trial at flowering in 2001. C. Oven bags filled with sterilised barley grain which were inoculated with 5 ml of pycnidiospore suspension of *L. maculans* and incubated on a bench in the laboratory. D. Barley grain colonised with *L. maculans*. E. Colonised barley grain spread around canola seedlings. F. Trap plants were sown weekly in the glasshouse (foreground) and returned to the glasshouse for incubation following exposure in the field (background). G. Trap plants in the field protected from animals with a wire cage. H. Automatic weather station (AWS).

5.3 RESULTS

5.3.1 Field trials

Kingsford trial 2000

Figure 5.2 shows the results of disease assessments for the Kingsford trial, which was sown on May 11, 2000. Infection was first observed on cotyledons of seedlings of all cultivars 27 days after sowing, on June 7 (Figure 5.2A). By June 15, 40-50% of seedlings of cvs Dunkeld, Hyola 42, Monty and Q2, had cotyledon infection and 17-30% of seedlings of cvs Pinnacle, Mystic, Scoop and Karoo had cotyledon infection. However, due to the variation between plots, this difference was not significant ($P>0.05$). Assessment of cotyledon infection was discontinued after June 15 due to senescence of cotyledons on seedlings of some cultivars. Leaf infection (leaf rating ≥ 1) was first observed on plants 35 days after sowing, on June 15, and by July 5, 100% of plants of all cultivars had leaf infection (Figure 5.2A). There was no significant difference ($P>0.05$) between the cultivars. The mean severity of leaf infection increased to a rating of approximately 1.4 for plants of all cultivars, and then seemed to plateau till August 16, after which leaf infection was no longer assessed (Figure 5.2B).

Stem infection (stem rating ≥ 1) was first observed on plants of cvs Hyola 42, Mystic and Q2 62 days after sowing, on July 12, and plants of all other cultivars developed stem infection by August 24, with plants of cv. Pinnacle taking the longest to develop stem symptoms (Figure 5.2A). Throughout the season, until the last assessment on November 1, the incidence of stem infection on plants of all cultivars increased in a linear fashion. On November 1, 40% of cv. Pinnacle plants had infected stems, significantly fewer ($P<0.05$) than all other cultivars which ranged from 67-97% of plants with infected stems. On both cvs Q2 and Monty, 97% of plants had developed stem infection, significantly more ($P<0.05$) than cvs Scoop, Dunkeld, Mystic and Pinnacle. Severity of stem infection followed a trend similar to that of disease incidence (Figure 5.2A&B). By the last assessment on November 1, the mean stem severity

of plants of cv. Pinnacle was 1.4, which was significantly less ($P<0.05$) than for all other cultivars, and the mean stem severity of plants of cv. Q2 was 5.5, which was significantly more ($P<0.05$) than for all other cultivars. For the other cultivars, mean stem severity ranged from 2.5 to 4.4.

Stem canker (stem rating ≥ 5) was first observed on July 12, in the form of crown canker on seedlings (Figure 5.2C), with 7% of plants of cv. Q2 affected. Over the next 6 weeks, 3% of plants of the cvs Hyola 42, Monty and Scoop were infected. Between August 16 and the last assessment on November 11, the incidence of stem canker on plants of cv. Q2 increased from 7% to 67%. Stem canker incidence on the other cultivars increased to between 23% and 27% for cvs Hyola 42, Karoo and Monty and to between 3% and 10% for cvs Pinnacle, Dunkeld and Scoop. No stem canker developed on plants of cv. Mystic. The incidence of stem canker on plants of cv. Q2 was significantly greater ($P<0.05$) than the incidence of stem canker on all other cultivars. The incidence of stem canker on plants of cvs Mystic and Pinnacle was significantly less ($P<0.05$) than the incidence of stem canker on cvs Hyola 42, Monty, Karoo and Q2.

Charlick trial 2000

Figure 5.3 shows the results of disease assessments on the Charlick trial, which was sown on May 10, 2000. Cotyledon infection was first observed on seedlings of all cultivars 28 days after sowing, on June 7 (Figure 5.3A). By June 15, incidence of cotyledon infection increased to between 60% and 87% of seedlings of all cultivars, with no significant difference ($P>0.05$) between them. Leaf infection was first observed on plants of all cultivars except cv. Dunkeld 36 days after sowing, on June 15, and incidence increased to 100% of plants of all cultivars by June 29 (Figure 5.3A). Severity of leaf infection increased to a plateau of 1.2 to 1.4 on

plants of all cultivars, and there was no significant difference ($P>0.05$) between them (Figure 5.3B).

Stem infection was first observed on plants of cv. Hyola 42 on July 13 (64 days after sowing) and by August 16 plants of all cultivars except Mystic and Dunkeld had stem infection (Figure 5.3A). Throughout the season, the incidence of plants with stem infection increased in a linear fashion on all cultivars. At the last assessment on November 1, the mean stem infection of cv. Pinnacle plants was 37%, significantly less ($P<0.05$) than all other cultivars and the stem infection of cv. Dunkeld plants was 57%, significantly less than cvs Scoop, Hyola 42, Monty, Karoo and Q2. All plants of cv. Q2 had developed stem infection, significantly more ($P<0.05$) than cvs Scoop, Mystic, Dunkeld and Pinnacle. Severity of stem infection increased in a linear fashion (Figure 5.3B). On November 1, the mean stem severity of plants of cv. Pinnacle was 1.4, which was significantly less ($P<0.05$) than all other cultivars except Dunkeld and the mean stem severity of plants of cv. Q2 was 5.8, significantly more ($P<0.05$) than all other cultivars. Again, mean stem severity ranged from 1.9 to 4.3 for the other cultivars.

Stem canker was first observed on July 12 in the form of crown canker on seedlings of cv. Hyola 42 (Figure 5.3C). By November 1, 77% of plants of cv. Q2 had developed stem canker, significantly more ($P<0.05$) than all other cultivars. Plants of cvs Dunkeld and Mystic did not develop stem canker and 10 - 37% of plants of other cultivars had stem canker.

Kingsford trial 2001

The results of disease assessments on the Kingsford trial, which was sown on May 31, 2001 are shown in Figure 5.5. Cotyledon infection was first observed on seedlings of cvs Dunkeld

Figure 5.2: Disease progress at weekly intervals, at the Kingsford trial site, 2000 (sown May 11). A. Mean incidence of *L. maculans* infection on cotyledons, leaves and stems, B. mean severity of infection on leaves (rating 1-4) and stems (rating 1-7) and C. mean incidence of stem canker. Cultivars; ♦ Dunkeld, ● Hyola 42, □ Karoo, ▲Monty, ○ Pinnacle, ■ Q2, Δ Scoop, × Mystic (------ cotyledon infection)

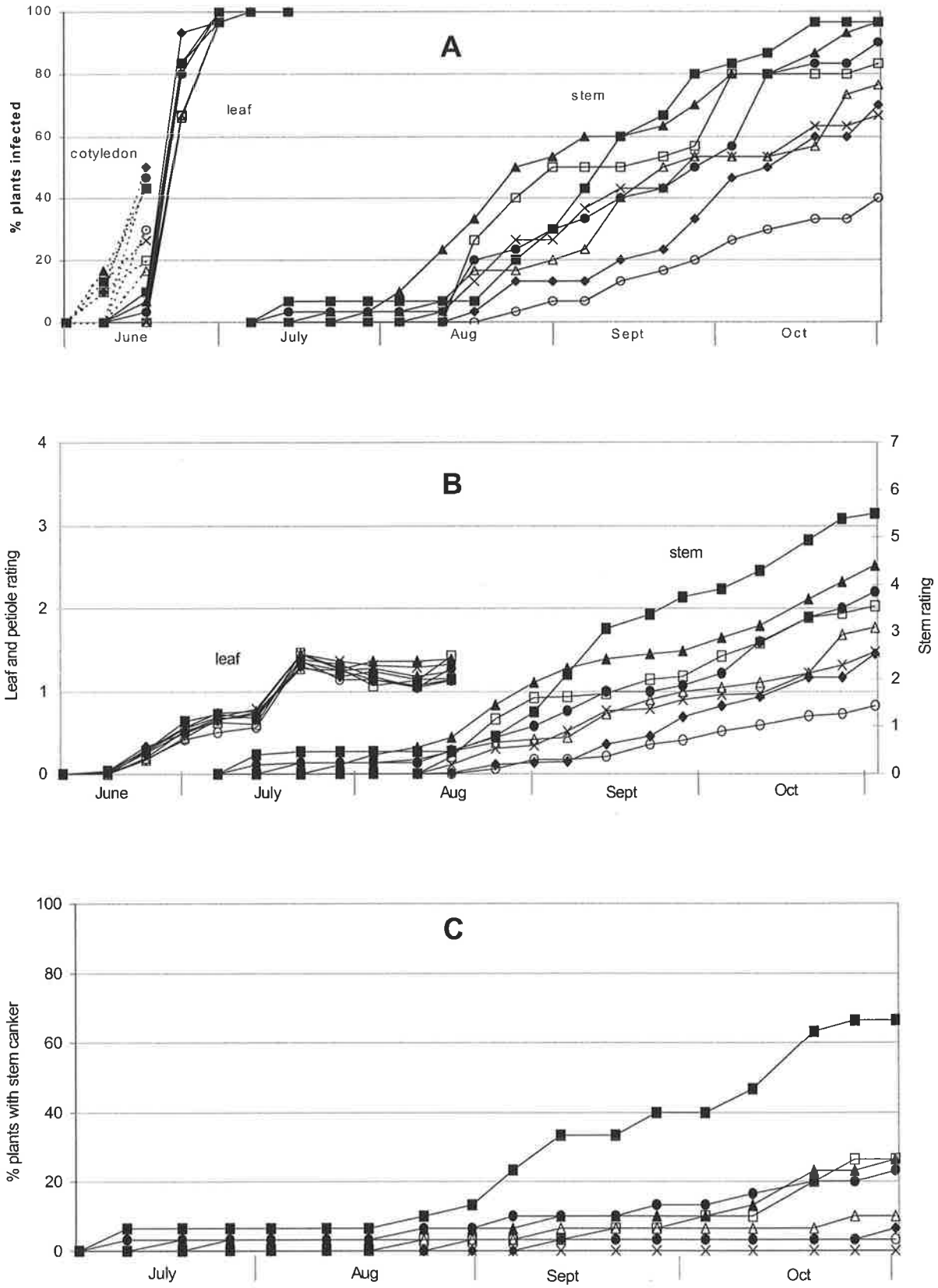
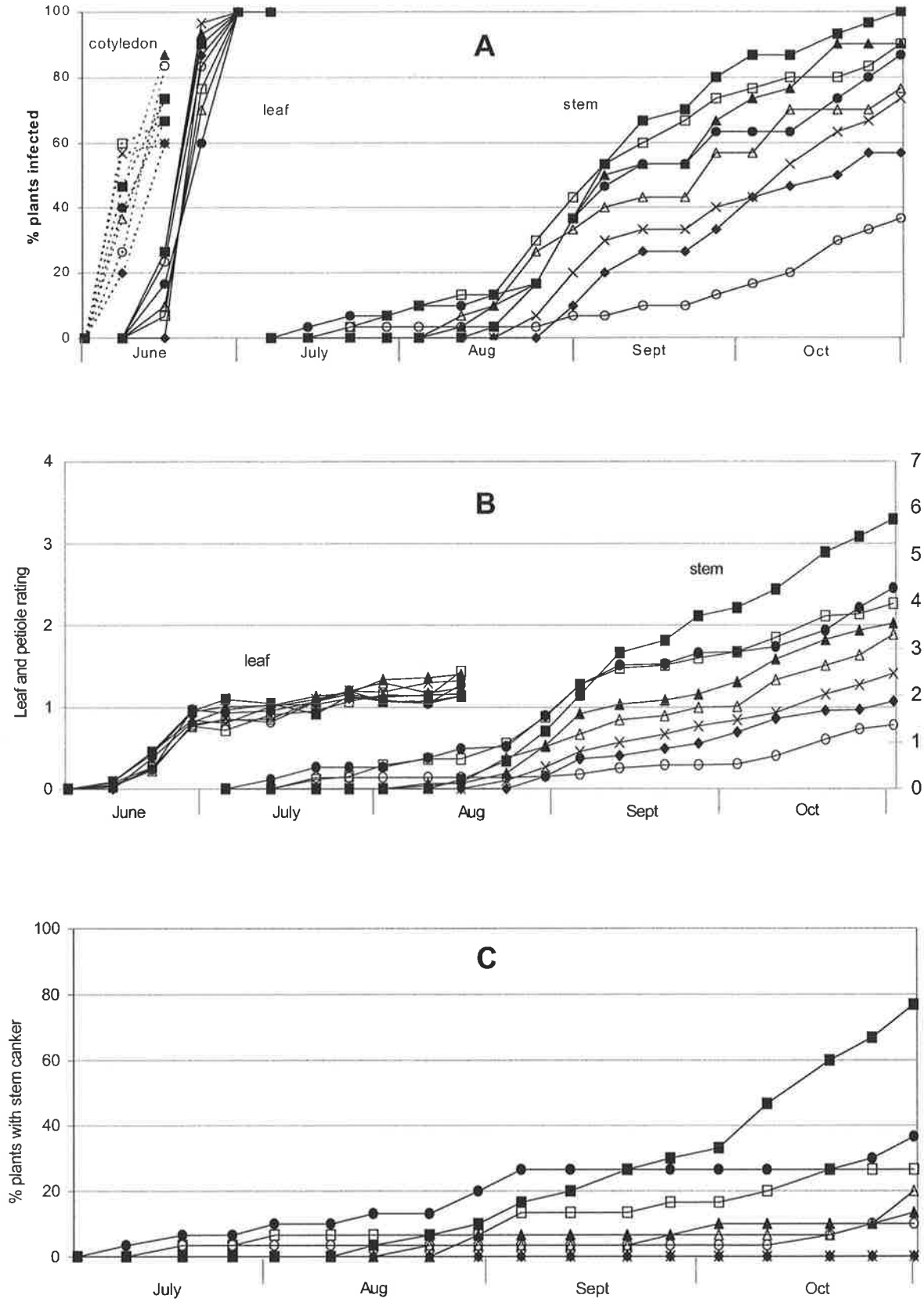


Figure 5.3: Disease progress at weekly intervals, at the Charlick trial site, 2000 (sown May 10). A. Mean incidence of *L. maculans* infection on cotyledons, leaves and stems, B. mean severity of infection on leaves (rating 1-4) and stems (rating 1-7) and C. mean incidence of stem canker. Cultivars; ♦ Dunkeld, ● Hyola 42, □ Karoo, ▲Monty, ○ Pinnacle, ■ Q2, Δ Scoop, × Mystic (------ cotyledon infection)



and Pinnacle on June 26, 26 days after sowing (Figure 5.5A). One week later, the incidence of cotyledon infection on all cultivars was 60-90% except for Hyola 60, which had an incidence of 4%, significantly less ($P<0.05$) than all other cultivars. Sixty-four percent of plants of cv. Hyola 42 had cotyledon infection, which was significantly lower ($P<0.05$) than that for cvs Scoop, Pinnacle and Mystic. Differences in cotyledon incidence among the other cultivars were not significant ($P>0.05$). Leaf infection was first observed on cv. Mystic on July 3, 33 days after sowing (Figure 5.5A). Six weeks later, all plants of all cultivars except Hyola 60, reached 100% incidence of leaf infection. Only 23% of cv. Hyola 60 plants developed leaf lesions, which was significantly less ($P<0.05$) than for all other cultivars. Infection on the leaves of cv. Hyola 60 appeared as small, restricted lesions with little or no pycnidia forming (Figure 5.4A), unlike the large lesions which formed abundant pycnidia on all other cultivars (Figure 5.4B). Since the mean severity of leaf infection for cultivars in 2000 did not add any more information than the incidence of leaf infection, the former was not calculated for 2001.

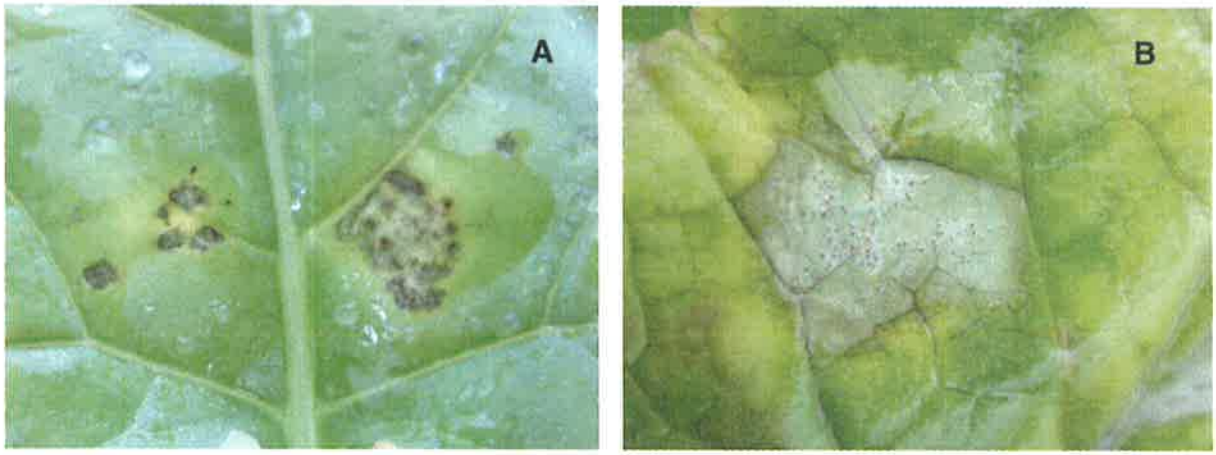


Figure 5.4: Small, restricted leaf lesions with no pycnidia on cv. Hyola 60 (A) compared with the large leaf lesions with abundant pycnidia formed on cv. Q2 (B).

Stem infection was first observed in cvs Q2, Karoo, Monty and Mystic on July 19, 49 days after sowing (Figure 5.5A). By August 9, incidence on those cultivars had increased to 27%, 20%, 13% and 3%, respectively, and all other cultivars had no stem infection. The incidence

of stem infection remained the same for the next 2 weeks, followed by another increase for all cultivars except Hyola 60. By October 17, the incidence of stem infection of plants of cv. Q2 had reached 100%, and the incidence of other cultivars ranged between 73% and 97%, except for cv. Hyola 60 which had an incidence of only 3% of plants with stem infection. By November 7, the incidence of stem infection of cvs Q2, Karoo and Monty was 100%, significantly more ($P<0.05$) than for cvs Scoop, Pinnacle and Hyola 60. Only 3% of plants of cv. Hyola 60 had infected stems, significantly less ($P<0.05$) than all other cultivars. Severity of stem infection increased in a linear fashion throughout the season (Figure 5.5B). The mean stem severity of plants of cv. Hyola 60 was only 0.03, significantly less ($P<0.05$) than for all other cultivars. The mean stem severity on cv. Pinnacle was 3.3, significantly less ($P<0.05$) than for all other cultivars except cvs Hyola 60 and Scoop. Mean stem severity ratings on cvs Q2, Hyola 42 and Karoo were between 5.9 and 6.2, significantly greater ($P<0.05$) than for all other cultivars.

Stem canker was first seen on plants of cvs Karoo, Q2, Monty and Mystic on July 25, 55 days after sowing (Figure 5.5C). The last cultivar to develop stem canker was Scoop, on September 12, and incidence continued to increase at different rates until the final assessment on November 7, except for cv. Hyola 60, which did not develop stem canker at all. Twenty percent of cv. Pinnacle plants developed stem canker by November 7, and together with cv. Pinnacle, both cultivars had significantly less ($P<0.05$) stem canker than all other cultivars. The incidence of stem canker on cvs Mystic and Scoop was 47% and 50% respectively, significantly less than that of cvs Monty, Hyola 42, Karoo and Q2, of which 73 - 93% of plants developed stem canker.

Charlick trial 2001

Disease assessment results on the Charlick trial, which was sown on May 25, 2001, are shown in Figure 5.6. Cotyledon infection was first noticed on plants of cvs Pinnacle, Scoop, Q2 and Monty on June 20, 26 days after sowing (Figure 5.6A). By July 3, the incidence of cotyledon infection was 60-87% on all cultivars except Hyola 60, which had an incidence of only 3%, significantly less ($P<0.05$) than for all other cultivars. There was no significant difference ($P>0.05$) in incidence of cotyledon infection among the other cultivars. Leaf infection was first observed on plants of all cultivars except cv. Hyola 60 on July 3, 39 days after sowing (Figure 5.6A). All cultivars reached a leaf infection incidence of 100% within 5 weeks, except for Hyola 60, which reached a maximum of only 7% of plants with leaf infection, significantly less ($P<0.05$) than for all other cultivars. Due to the senescence of leaves, the incidence of leaf infection on Hyola 60 was reduced to 3% by August 15.

Stem infection was first observed in cvs Q2 and Monty on July 11, 47 days after sowing (Figure 5.6A). By July 25, 17% of plants of cv. Q2 had stem infection and all other cultivars had up to 3% of plants with stem infection. Over the following 4 weeks, there was little increase in stem infection but from September 5 until the last assessment on October 31, the incidence of stem infection on cv. Q2 inreased rapidly to 100%, significantly greater ($P<0.05$) than for all other cultivars except cv. Monty. No stem infection was observed in plants of cv. Hyola 60 by October 31. The incidence of stem infection of other cultivars ranged between 30% and 77%. The severity of stem infection on all cultivars remained below 1.5 until early in September, after which the severity increased in a linear fashion until November 7 (Figure 5.6B). The mean stem severity of cv. Q2 reached 6.3 by November 7, significantly higher than for all other cultivars. The mean stem severity rating on plants of cv. Hyola 60 was 0. The mean stem severity of other cultivars ranged between 1.2 and 3.5.

Figure 5.5: Disease progress at weekly intervals, at the Kingsford trial site, 2001 (sown May 31). A. Mean incidence of *L. maculans* infection of cotyledons, leaves and stems, B. mean severity of infection on stems (rating 1-7) and C. mean incidence of stem canker. Cultivars; ♦ Dunkeld, ● Hyola 42, □ Karoo, ▲Monty, ○ Pinnacle, ■ Q2, Δ Scoop, × Mystic, ---◊--- Hyola 60.

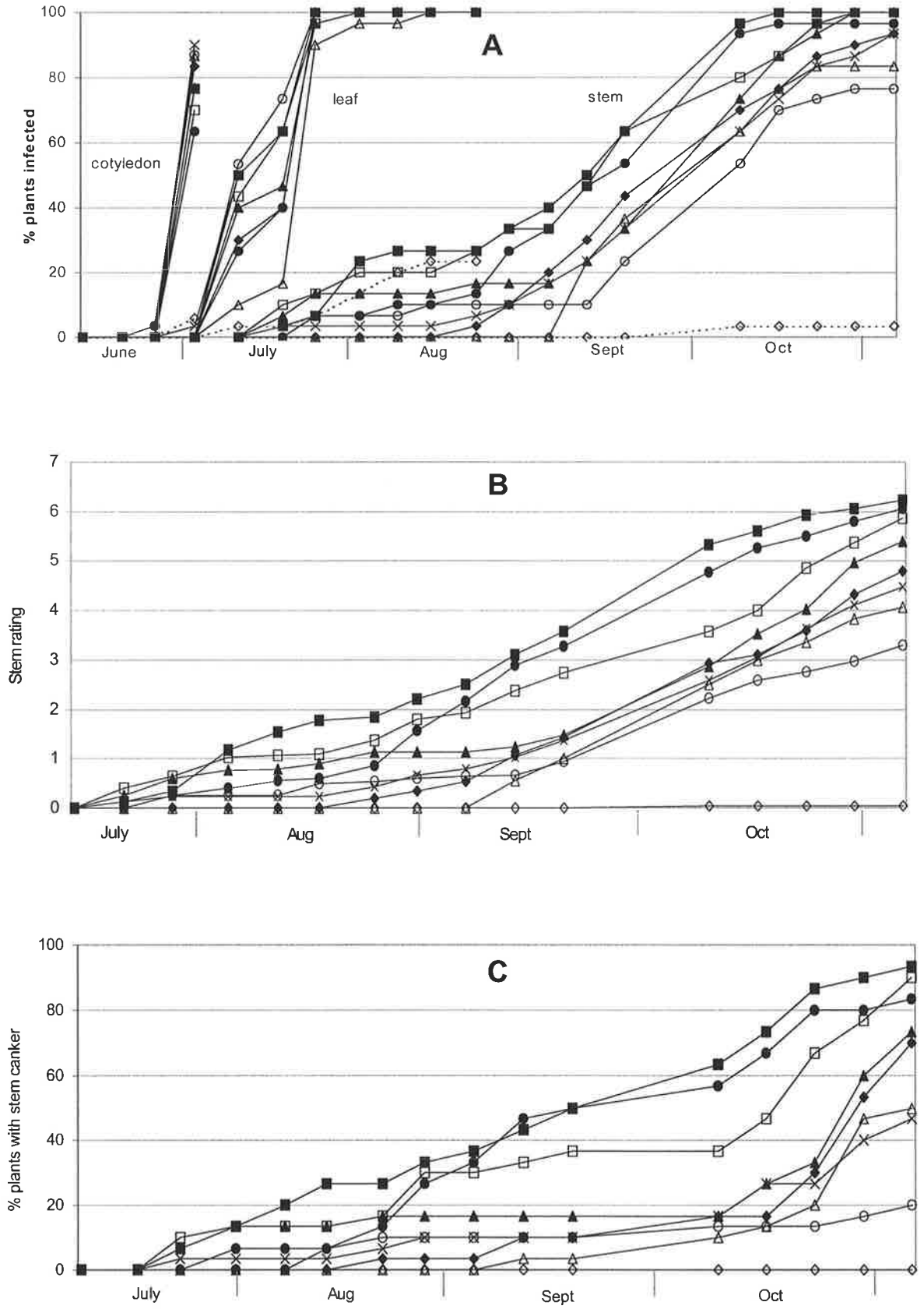
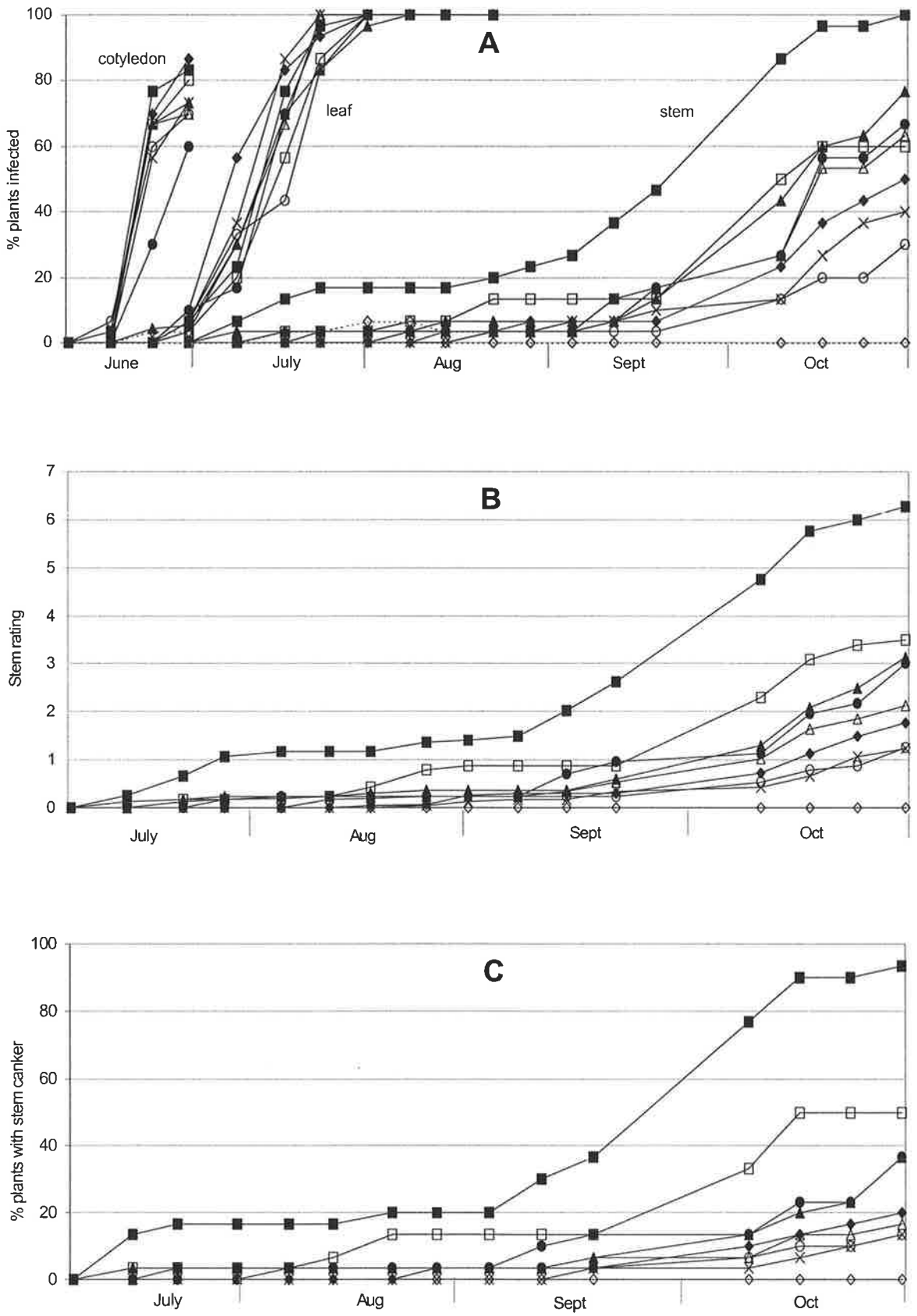


Figure 5.6: Disease progress at weekly intervals, at the Charlick trial site, 2001 (sown May 25). A. Mean incidence of *L. maculans* infection of cotyledons, leaves and stems, B. mean severity of infection on stems (rating 1-7) and C. mean incidence of stem canker. Cultivars; ♦ Dunkeld, ● Hyola 42, □ Karoo, ▲Monty, ○ Pinnacle, ■ Q2, Δ Scoop, × Mystic, ---◊--- Hyola 60.



Stem canker was first seen on plants of cvs Q2, Monty and Karoo on July 19, 55 days after sowing (Figure 5.6C). Over the following 7 weeks, the incidence of stem canker remained almost the same for most cultivars, and the incidence on plants of cv. Q2 increased to 93% by November 7, which was significantly greater ($P<0.05$) than for all other cultivars. The incidence of stem canker on cvs Karoo, Hyola 42 and Monty reached 37 - 50%, significantly greater ($P<0.05$) than for cvs Scoop, Pinnacle, Mystic and Hyola 60, for which up to 17% of plants developed stem canker.

5.3.2 Weather data

Daily weather data collected between the sowing date and the last date of assessment are presented graphically for each trial in Appendices 8 to 11. A summary of the data, averaged from sowing to 1) the last cotyledon rating, 2) the last leaf rating and 3) the last stem rating of each trial, is shown in Table 5.1. In general, the average temperature was slightly higher at Charlick than at Kingsford for each period. Cumulative degree-days were greater at Charlick than at Kingsford in each year for all periods, but similar for 2000 and 2001 up to the last cotyledon rating and stem rating, and greater in 2001 than 2000 up to the last leaf rating. The average relative humidity was higher at Kingsford than at Charlick during all periods, but was similar for the two years in all periods. The average leaf wetness was lower at Kingsford than at Charlick except up to the last leaf rating in 2001. Cumulative rainfall was greater at Charlick than at Kingsford in 2000, and in 2001 the cumulative rainfall was also greater at Charlick than at Kingsford for sowing to last cotyledon rating but, as the season progressed, cumulative rainfall became greater at Kingsford than Charlick.

Table 5.1: Average (ave) and cumulative (cum) climate data, during different periods for each trial. Cumulative degree-days were calculated by adding the daily mean temperatures.

	Kingsford 2000	Charlick 2000	Kingsford 2001	Charlick 2001
1. Sowing – last cotyledon rating				
Ave min temp (°C)	7.3	8.3	8.2	8.1
Ave mean temp (°C)	11.4	12.2	11.4	12.0
Ave max temp (°C)	15.8	16.5	15.8	16.2
Cum degree days	400.4	440.2	363.4	457.4
Ave min RH (%)	60.1	61.1	61.5	63.9
Ave mean RH (%)	81.0	78.5	81.8	78.6
Ave max RH (%)	93.4	89.4	93.6	89.0
Ave LW (%)	27.9	40.7	23.3	30.3
Cum rainfall (mm)	50.6	69.2	44.4	48.4
2. Sowing – last leaf rating				
Ave min temp (°C)	7.8	8.9	7.2	7.2
Ave mean temp (°C)	11.9	12.8	11.0	11.7
Ave max temp (°C)	16.2	16.7	15.7	16.1
Cum degree days	333.8	370.2	854.6	979.1
Ave min RH (%)	59.7	61.8	59.4	61.6
Ave mean RH (%)	80.1	77.6	80.5	77.4
Ave max RH (%)	92.3	88.2	93.3	88.4
Ave LW (%)	38.6	47.1	29.3	29.1
Cum rainfall (mm)	50.0	62.2	102.8	90.8
3. Sowing – last stem rating				
Ave min temp (°C)	7.2	7.3	7.7	7.6
Ave mean temp (°C)	11.5	12.0	11.8	12.4
Ave max temp (°C)	16.7	17.5	16.9	17.8
Cum degree-days	2020.0	2120.7	1896.9	2063.7
Ave min RH (%)	55.6	51.8	57.6	54.1
Ave mean RH (%)	80.7	76.5	81.1	75.6
Ave max RH (%)	95.2	91.7	94.6	89.4
Ave LW (%)	27.1	31.4	27.6	33.1
Cum rainfall (mm)	279.6	360.4	271.8	244.8

min – minimum, max – maximum, temp – temperature, RH – relative humidity, LW – leaf wetness

5.3.3 Spore trap plants

Appendix 12 lists the mean number of leaf lesions detected on spore trap plants for each week at the two trial sites in both years. Figures 5.7 to 5.10 show the amount of infection that occurred each week throughout the season for each trial, along with the weekly mean temperature, total rainfall, mean wind speed and direction.

In 2000, the first set of trap plants (May 31-June 5) at both Kingsford and Charlick yielded 14.1 and 18.2 leaf lesions/plant, respectively. At Kingsford, infection reached a maximum of 37.3 lesions/plant in the week of June 22-28, and then it fluctuated between 1.9 and 12.6 until

September 12, after which no infection was detected (Figure 5.7). At Charlick, infection of trap plants was more consistent over time than Kingsford. Infection ranged between 12.4 and 25.8 leaf lesions/plant until July 28, and then infection dropped to between 2.7 and 6.4 leaf lesions/plant from July 29 until September 13, except for the week of August 3-10 when infection reached 19.3 (Figure 5.8). After September 13, no infection was detected. At both locations, it was evident that during weeks when infection of spore trap plants was common, the total weekly rainfall, mean weekly temperature and wind activity also increased. This apparent correlation, however, occurred only during June, July and to a lesser extent August, but during September and October trap plants did not become infected, even when rainfall, temperature and wind activity were high.

In 2001, at both locations, the first set of spore trap plants remained healthy but the second set exposed, during the week of May 23-29, had a small amount of infection. At Kingsford, infection reached a maximum of 32.9 during the week of August 2-8, before which time only 0.2 - 14.0 leaf lesions/plant were detected (Figure 5.9). After this, infection fluctuated, declining to 5.7 leaf lesions/plant during the week of September 5-11, and then remained under 0.5 leaf lesions/plant until October 16, after which no infection was detected. At Charlick, infection remained under 6.8 leaf lesions/plant until the week of August 30-September 5, where infection was a maximum of 14.9 leaf lesions/plant (Figure 5.10). There were fewer than 1.1 leaf lesions/plant until October 10, after which no infection occurred. An apparent correlation was observed between increases in total weekly rainfall, mean weekly temperature, wind activity and infection of spore trap plants between June and August at Kingsford.

Figure 5.7: Total rainfall (mm), mean temperature (°C), wind speed (km/h), wind direction and number of leaf lesions on Hyola 42 trap plants at Kingsford each week throughout the 2000 growing season.

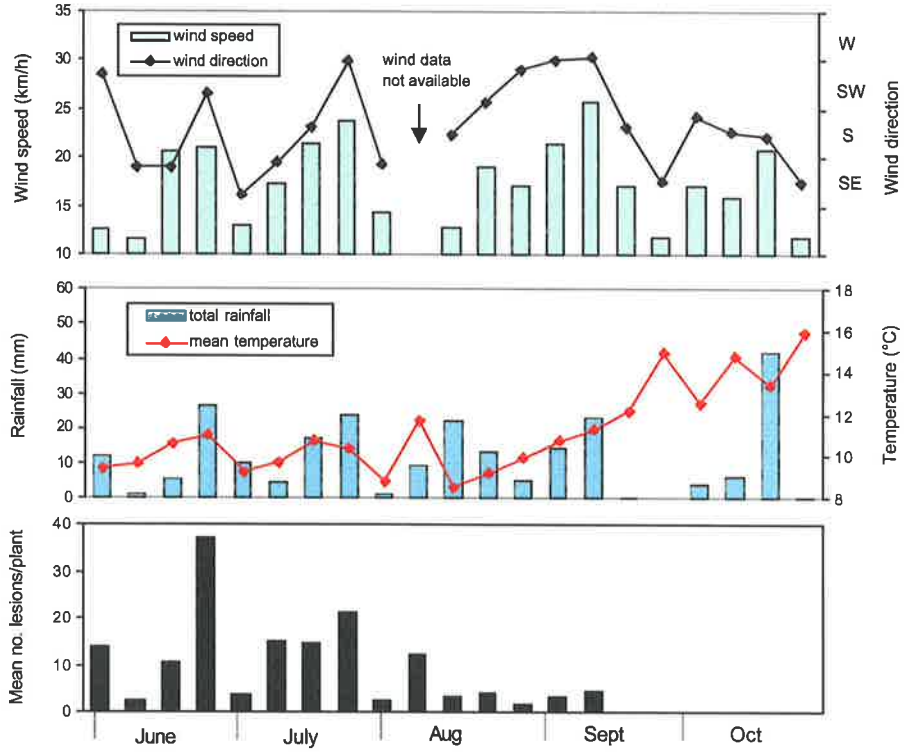


Figure 5.8: Total rainfall (mm), mean temperature (°C), wind speed (km/h), wind direction and number of leaf lesions on Hyola 42 trap plants at Charlick each week throughout the 2000 growing season.

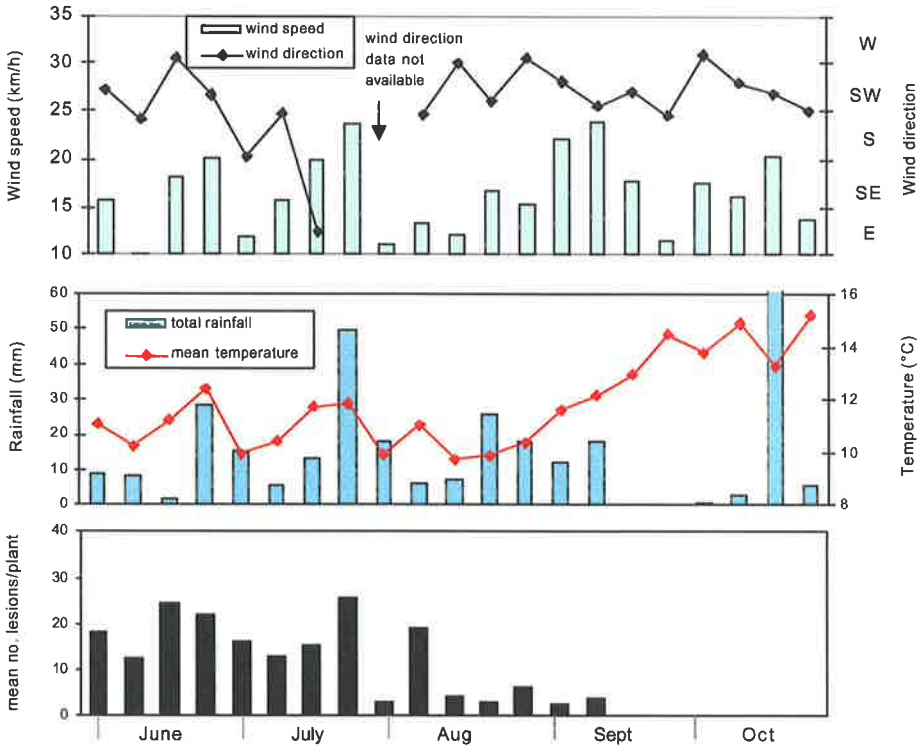


Figure 5.9: Total rainfall (mm), mean temperature (°C), wind speed (km/h), wind direction and number of leaf lesions on Hyola 42 trap plants at Kingsford each week throughout the 2001 growing season.

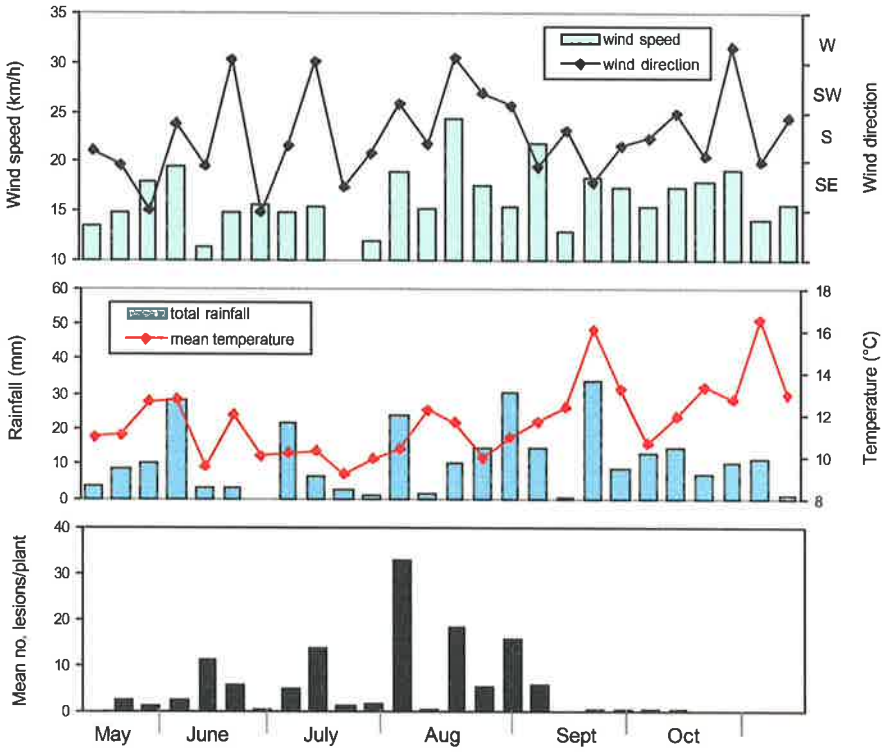
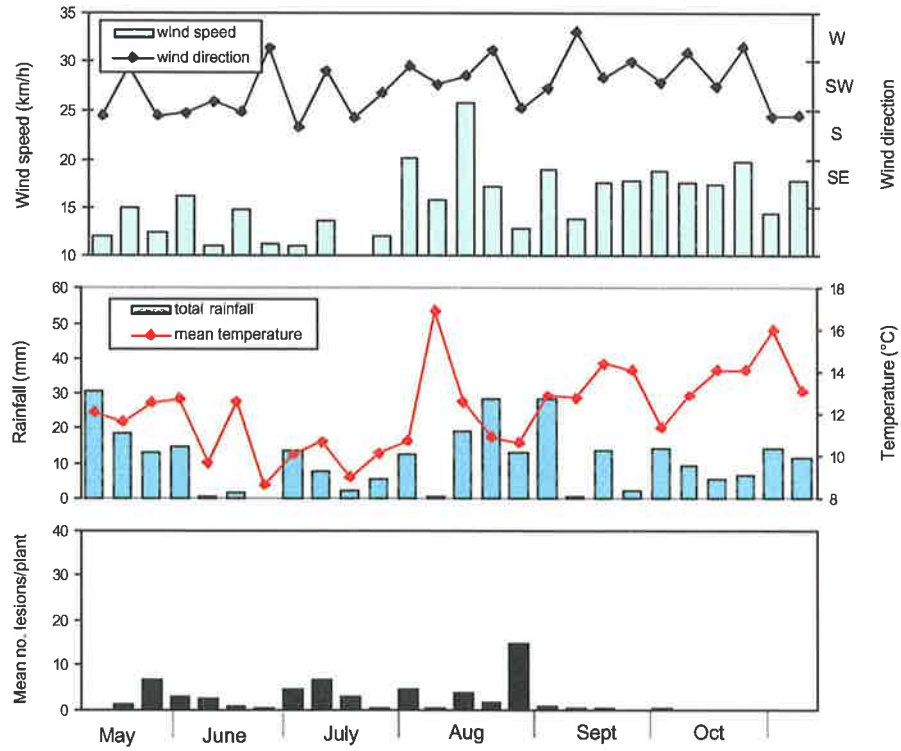


Figure 5.10: Total rainfall (mm), mean temperature (°C), wind speed (km/h), wind direction and number of leaf lesions on Hyola 42 trap plants at Charlick each week throughout the 2001 growing season.



The total number of leaf lesions produced on spore trap plants throughout the season for each trial is shown in Appendix 12. Overall, ascospore-derived infection was greater in 2000 than in 2001, even thought trap plants were not placed at field trial sites prior to May 31 in 2000. In 2001 the infection was much less at Charlick than at Kingsford.

5.3.4 Disease modelling

5.3.4.1 Effect of disease on yield

At harvest, yields were calculated for three of the trials, but the trial at Charlick in 2000 was not harvested due to severe pod damage by birds. At Kingsford, yield data from both years showed no effect of replication ($P>0.05$) and there were significant differences ($P<0.05$) between cultivars. At Charlick in 2001, however, all three replications were significantly different ($P<0.05$) from each other, so data were not used in subsequent analyses.

Yields from the Kingsford trials were plotted against a number of different disease incidence and severity ratings at different times throughout the season (Figure 5.11) and yield and disease data can be found in Appendix 13. The plots show that cotyledon infection in June, stem infection in August, stem infection in November and stem severity in November were all poorly correlated with yield ($R^2 < 0.2$). Stem canker in August and November was correlated with yield, with R^2 values of 0.57 and 0.53, respectively. The incidence of stem canker ranged from 0 to 50% in August and from 0 to 100% in November.

Figure 5.12 shows the correlations between incidence of stem canker and yield for each cultivar individually, at the Kingsford trial site in 2000 and 2001. Yields for cv. Dunkeld were high (approximately 3.0 t/ha) and they were not affected by the incidence of stem canker, since the regression line is almost vertical. The yield of cv. Hyola 42 ranged between 0.5 and 3.0 t/ha, and decreased when the incidence of stem canker increased. The yields of

cvs Karoo and Monty were 1.0 - 2.0 t/ha and 1.5 - 3.0 t/ha, respectively, and both were affected by changes in the incidence of stem canker. The yields of cvs Mystic and Scoop ranged between 2.0 and 3.0 t/ha, and were poorly correlated with the incidence of stem canker (R^2 = 0.39 and 0.09, respectively). Stem canker incidence on cv. Pinnacle was very low and so had little effect on its relatively high yield. In contrast, the high incidence of stem canker on cv. Q2 had an effect on yield. In summary, yield and stem canker incidence were correlated in susceptible cultivars but not in resistant cultivars.

Based on a model of blackspot in field peas (A. Schoeny, personal communication, 2002) and using linear regression analysis in Statistix®, a predictive equation for the effect of incidence of stem canker in November (SCN) on yield of susceptible cultivars can be written as:

$$\text{Yield (t/ha)} = c - v\,\text{SCN(\%)}$$

Yield can be calculated for the following cultvars using the variables (*c* and *v*):

	Hyola 42	Karoo	Monty	Q2
c	2.985	2.183	3.223	2.791
v	0.0204	0.0123	0.0164	0.0191

As linear regressions between yield and stem canker were not significant (P>0.05) for the more resistant cultivars, predictive equations were not formulated.

Figure 5.11: Correlations between yield and disease incidence on all cultivars at the Kingsford trials in 2000 and 2001 at different stages of the growing season. A. cotyledon infection in June, B. stem infection and C. stem canker in August, D. stem rating, E. stem infection and F. stem canker in November.

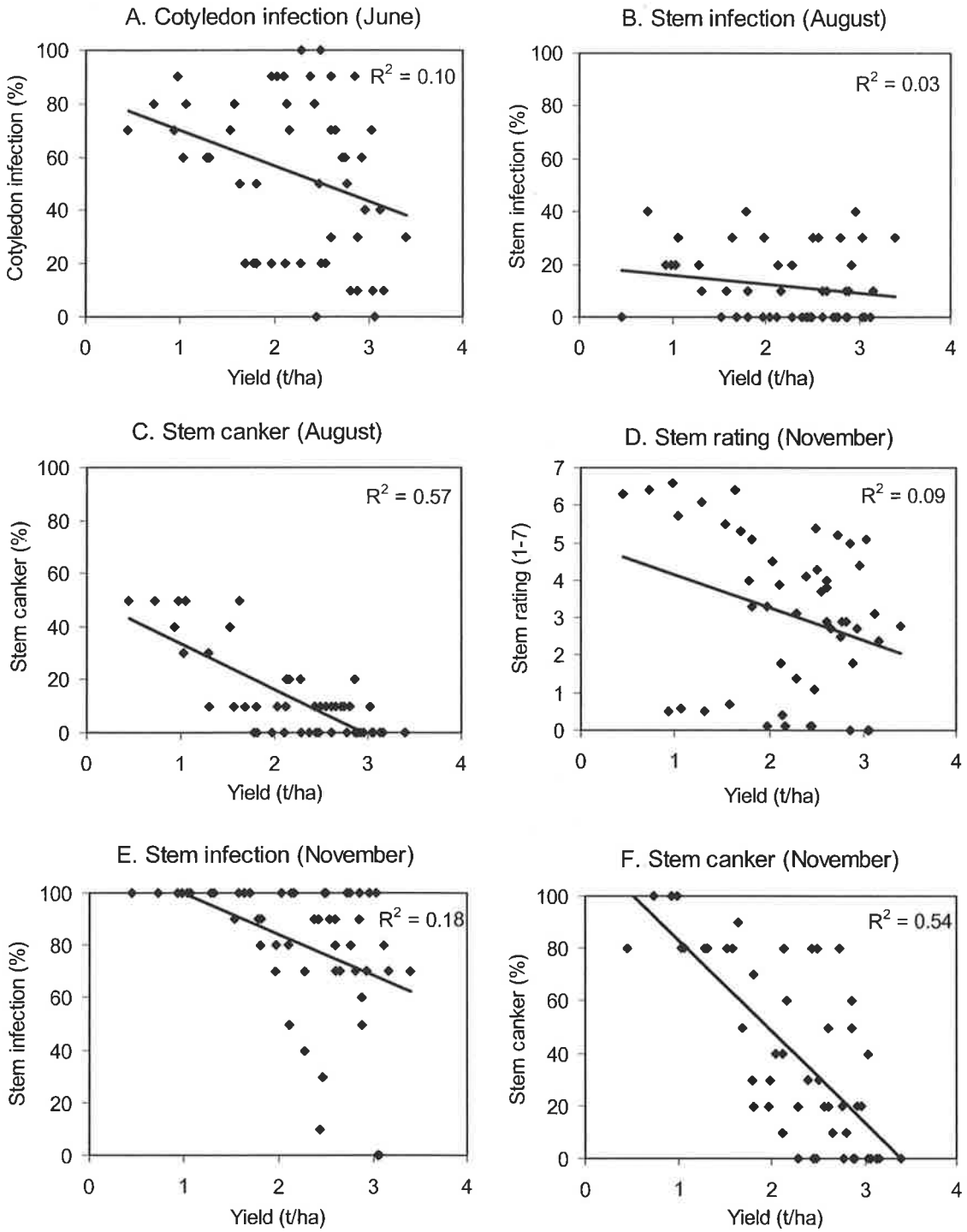
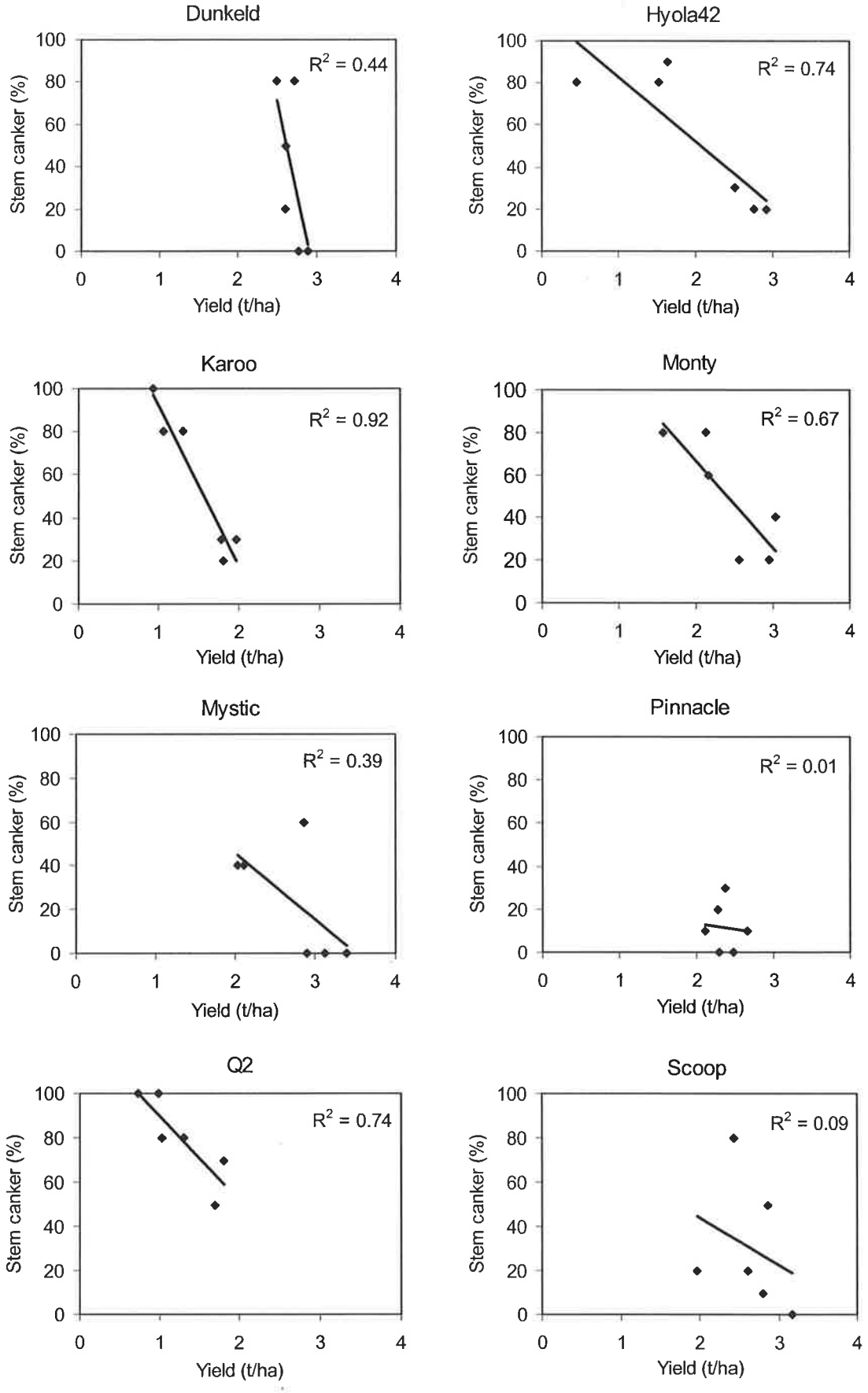


Figure 5.12: Correlations between incidence of stem canker in November and yield for each cultivar at the Kingsford trial sites in 2000 and 2001. Hyola 60 is not shown, as stem canker was negligible.



5.3.4.2 Effect of weather variables and cultivar on disease

As yield was correlated most closely with incidence of stem canker in November (section 5.3.4.1), the effects of climatic variables on stem canker incidence were investigated. Weather variables which were considered most likely to influence blackleg disease epidemics are listed in Table 5.2.

Table 5.2: Climatic variables selected to assess correlations with stem canker incidence. Pre-sowing is the period from Jan 1 to the sowing date, season is the period from the sowing date till the date of last assessment and leaf growth is the period from the first leaf (GS 2.1) till plants begin to elongate (GS 3.1).

	Kingsford 2000	Charlick 2000	Kingsford 2001	Charlick 2001
Cum rainfall – pre-sowing (mm)	161.2	165.2	93.5	111.9
Cum rainfall – season (mm)	279.6	360.4	271.8	230.6
Cum DD – season	2002.1	2102.9	1884	1936
Ave WS – season (km/h)	17.7	16.6	16.4	15.5
Ave LW – season (%)	27.1	31.4	27.6	33.8
Ave RH – season (%)	80.7	76.5	81.1	75.8
Ave WS – leaf growth (km/h)	17.6	16.1	13.0	12.5
Ave LW – leaf growth (%)	21.6	36.9	28.4	32.4
Ave RH – leaf growth (%)	83.0	78.8	84.1	81.5
Cum rainfall – leaf growth (mm)	100.4	120.4	77.2	72
Ave temp – leaf growth (°C)	10.3	11	10.2	11.3
Cum DD – leaf growth	588.1	549.7	518.8	566.6
Spore trap infection – leaf growth	98.7	147	39.5	20.8
No. of days temp > 15°C – leaf growth	13	19	11	18
No. of days max LW = 100% – leaf growth	36	47	40	49

Cum – cumulative, ave – average, DD – degree days, WS – wind speed, LW – leaf wetness, RH – relative humidity, temp – temperature, max – maximum.

Mr. Andreas Kiermeier (BiometricsSA) carried out the following statistical analysis using "R" (Ihaka and Gentleman 1996). Stem canker incidence was converted to a proportion (p), between 0 and 1, so that in each plot, 0 indicated that no plants had stem canker and 1 indicated that all plants had stem canker. Due to the binary nature of the data, a generalised linear model (GLM) was used, in which the proportions were transformed using a logit transformation.

$$\pi = \log \frac{p}{1-p}$$

The GLM attempts to explain the transformed responses (π) through a linear predictor containing the climatic variables, with the minimum model containing only cultivar in the linear predictor. A stepwise approach was then used to add or remove climatic variables from the model until a final model was chosen which explained as much of the variablility as possible.

The final model involved the following variables; cultivar, cumulative rainfall pre-sowing (C), interaction between cultivar and cumulative rainfall pre-sowing, degree days during leaf growth (L) and degree days for the season (S). A predictive equation can be written as:

$$\pi = a + b\,C + c\,L + d\,S$$

The variables a and b are different for each cultivar as follows:

	Dunkeld	Hyola 42	Karoo	Monty	Mystic	Pinnacle	Q2	Scoop
a	30.05	27.63	28.98	27.81	30.48	24.20	30.58	25.70
b	-0.026	0.005	-0.004	0.001	-0.039	0.016	-0.001	0.012

The constants are c = -0.025 and d = -0.007.

The actual proportions can then be calculated using the following equation:

$$p = \log \frac{\exp\,(a + b\,C + c\,L + d\,S)}{1 + \exp\,(a + b\,C + c\,L + d\,S)}$$

The fitted vs residual plot (Appendix 14) showed that the model fitted well and the residual deviance of the final model (99.43) was reasonably close to the degrees of freedom (78), which is what would be expected for a reasonable fit.

5.4 DISCUSSION

5.4.1 Disease progression

Field trials revealed that the progression of blackleg in canola plants varied for different cultivars and weather conditions.

The incidence of cotyledon infection for cultivars other than Hyola 60 ranged from 60 to 90% for all trials except at Kingsford in 2000, when it ranged from 15 to 50%. There was no apparent difference in weather conditions during the cotyledon growth stage at Kingsford in 2000 compared to the other trials. The difference in cotyledon infection may be due to lower levels of ascospore inoculum at Kingsford in 2000 than at the other trial sites. Exposure of trap plants from the time of sowing would have enabled confirmation of this. As the same amount of barley grain inoculum, containing mycelia and pycnidia, was spread over plots at each trial site, it probably did not contribute to the difference. The difference between cultivars for incidence of cotyledon infection was not significant, except for cv. Hyola 60, which developed very few cotyledon lesions, possibly due to an unidentified anti-fungal compound which restricted spore germination and hyphal growth (section 4.3.1).

The incidence of leaf infection reached 100% for all cultivars, except for cv. Hyola 60. From the first observation of leaf infection, however, it took 2-3 weeks for 100% of plants to develop leaf infection in 2000 and 5-6 weeks in 2001. This delay in 2001 was most likely due to the lower mean temperature (approximately 1°C less) and mean leaf wetness (approximately 14% less) during leaf growth compared to 2000 (Table 5.1). In 2000, the severity of leaf infection reached a plateau because older leaves were senescing as new leaves began to express symptoms, which resulted in a constant mean severity. It was evident that, except for cv. Hyola 60, all cultivars tested were susceptible to leaf infection. This contradicts earlier studies in Australia (Roy 1984) and abroad (Mithen *et al.* 1987; Annis and

Goodwin 1997b) which suggested that leaf resistance existed in other cultivars of oilseed rape. In Australia, the present population of *L. maculans* is possibly more virulent than two decades ago in this country, and presently in Europe and Canada (section 2.6.5), based on ratings of blackleg susceptibility (Table 3.1). As most current cultivars, which are susceptible to leaf infection, have been developed from earlier cultivars, which expressed leaf resistance, it is possible that this resistance has broken down. The low level of infection observed in the leaves and cotyledons of cv. Hyola 60 in field trials supports findings using the scanning electron microscope that cv. Hyola 60 was able to restrict germination of spores and hyphal growth on the leaf surface (section 4.3.1).

Stem infection and canker were variable across the range of cultivars tested, with rankings generally consistent over the four trials. In the two trials in 2001, stem infection was observed in only 3% of plants of cv. Hyola 60 at Kingsford and there was no stem infection in this cultivar at Charlick. Cv. Pinnacle appeared to be the next most resistant cultivar in all four trials, with cvs Mystic, Dunkeld and Scoop also showing some resistance to stem infection. The extent of infection in these cultivars varied between trials, most likely due to differences in weather. As this is a complex interaction, it is difficult to determine the specific conditions which affect infection of each cultivar, but this can be achieved by modelling the data (section 5.3.4.2). The Canadian cv. Q2 was the most susceptible in all four trials, reaching 100% stem infection incidence in three trials and 97% at Kingsford in 2000. The cvs Hyola 42, Karoo and Monty were also susceptible to blackleg in all four trials. These results were generally consistent with the published blackleg resistance ratings listed in Table 3.1. However, cv. Pinnacle expressed more resistance to stem infection than all other cultivars except cv. Hyola 60 in these trials, but the previous ratings suggested that cv. Pinnacle was less resistant than cvs Dunkeld, Mystic, Scoop and Oscar. One reason for this may be that cv. Pinnacle responded differently to blackleg infection in these particular

environments compared to disease nursery sites in other states of Australia, where cultivars are monitored for blackleg resistance by the National Brassica Improvement Program. Therefore, for more accurate assessment of cultivar performance, disease nurseries should be located in a more diverse range of areas and, possibly, ratings should be given on a regional basis. In disease nurseries in WA, plants are rated for stem canker in addition to survival counts, which are carried out nationally, and a WA blackleg rating guide is published (Khangura *et al.* 2002).

Field trials revealed that cotyledon and leaf infection were not correlated with stem infection. This was expected, as resistance reactions in adult plants to stem infection are thought to have a different genetic basis to cotyledon and leaf resistance in seedlings (Rimmer and van den Berg 1992). Restriction of cotyledon and leaf infection in cv. Hyola 60 reduced hyphal growth into the stem, and allowed more efficient stem resistance and hence decreased severity of stem infection. Therefore, it is important to incorporate leaf resistance into breeding programs, along with stem resistance, to produce a more robust, polygenic resistance which is less likely to be broken down by the sexually reproducing *L. maculans*. Pacific Seed, which produced Hyola 60 (Buzza and Easton 2002), has now achieved this.

5.4.2 Ascospore-derived infection

By exposing canola plants for 1-week periods throughout the growing season, at least 20 m from each trial, the amount of infection due to ascospore release from stubble in nearby fields was estimated. Infection of spore trap plants due to pycnidiospores from the trial itself was considered to be highly unlikely, as pycnidiospores have been shown to be dispersed up to 1 m from the pycnidia (Barbetti 1976). Furthermore, no infection was detected in October when pycnidiospores were present on leaves and stems and conditions were conducive for dispersal and infection.

Spore trap plants in 2001 showed that ascospore-derived infection at each trial site occurred from May until October, whereas trap plants were not placed in the field until the first week of June in 2000, so we were unable to establish when the plants were first exposed to naturally occurring ascospore inoculum. The last time in which infection occurred was in the second week of September. Therefore, in this study ascospore-derived infection began towards the end of autumn, was greatest during winter, and ended mid-way through spring, observations which confirm findings by McGee (1977). In the northern hemisphere, timing of ascospore release varies (section 2.4.2), but it often coincides with the period of crop growth (Hall 1992). At field trial sites, the level of ascospore-derived infection fluctuated between 0 and 32.9 leaf lesions per plant from June to October. Ascospore-derived infection was very low throughout the season at Charlick in 2001, possibly due to the weather conditions leading up to and/or during the season, such as low cumulative rainfall between May and October compared to all other trials (Table 5.1). This low ascosporic infection may also be associated with the low stem disease severity recorded at Charlick 2001 (Figure 5.6B) compared to the other trials. At both trial sites in 2000, infection of spore trap plants was greatest during June and July, whereas the majority of infections at both trial sites in 2001 occurred during August. This may be due to the greater cumulative rainfall between January and sowing in 2000 compared to 2001, which may have have hastened maturation of pseudothecia and ascospore release (Petrie 1994; Pérès *et al.* 1999).

Ascospore-derived infection was correlated closely with mean temperature, cumulative rainfall, wind speed and wind direction. At both sites, north-westerly winds are associated with low pressure fronts which result in high wind speed and rainfall. South-easterly winds occur towards the end of low pressure systems and are generally followed by calm, dry conditions. As ascospore release requires moisture (McGee 1977) and ascospore germination

requires at least 48 h of leaf wetness (Biddulph *et al.* 1999), periods of increased rainfall are likely to encourage ascospore-derived infection. Furthermore, since ascospore germination is maximal at 20°C and spread of leaf lesions is most rapid between 15°C and 20°C (Toscano-Underwood *et al.* 2001), temperatures closest to these optima will promote ascospore-derived infection. Therefore, it is possible to determine when ascospore showers occur during the season and how rapidly infection will develop, which may aid growers with management of the disease. In WA, a model has been developed to predict maturation of pseudothecia and ascospore release based on daily rainfall and temperature for the whole year (Salam *et al.* 2002). Using local weather data from Kingsford and Charlick, preliminary results showed that the WA model model was able to predict when ascospore infection would occur at the two trial sites used in this study (M. Salam, unpublished data).

As canola crops are usually sown in May and June in southern Australia, seedling emergence coincides with the period of greatest potential for ascospore-derived infection, when the plant is most vulnerable to infection (section 5.4.1). McGee and Emmett (1977) showed that by delaying sowing, yield loss from disease was reduced, but yield was lower due to the shorter season. However, the lower yield due to the short season was significantly less than the yield lost when an epidemic occurred. Khangura and Barbetti (2001) found that sowing date had no effect on disease in WA, however, their data were gathered from crops sown by commercial growers, who were not able to take into account predictions of ascospore release. With the information gathered from spore trap plants and the model that is currently being developed in WA, it appears that delayed sowing when ascospore release is expected to occur early would reduce yield loss to blackleg. However, if ascospore release were expected to be late, then delayed sowing would not reduce yield loss to the disease.

5.4.3 Disease modelling

Data collected in field trials were analysed using multiple linear regression. This revealed that blackleg stem canker reduced yield in cultivars with low levels of resistance. Furthermore, cultivar, rainfall and temperature influenced the incidence of stem canker. However, these relationships have been identified using a limited set of data, which produced a large amount of variability. They will need to be validated through the collection and analysis of data obtained from a large number of field trials from a range of different growing regions using as many commercial cultivars as possible.

Yield loss has been associated with blackleg stem canker (Zhou *et al.* 1999; Khangura and Barbetti 2001) and losses are usually up to 10%, but can reach as much as 30-50% (Hall *et al.* 1993; Barbetti and Khangura 1999; Zhou *et al.* 1999). In this study, linear regression analysis showed that yield was poorly correlated with incidence of cotyledon and stem infection as well as disease severity on the stem. However, a correlation of $R^2 = 0.53$ for yield and stem canker showed that this symptom of the disease was most closely correlated to yield, as was found by Zhou *et al.* (1999). The regression analysis revealed that a 10% increase in stem canker would reduce yield by 10% but this estimate would need to be tested further using a larger set of data and trials including plots treated with fungicides in order to obtain a more accurate prediction. Cotyledon infection had little influence on yield. However, in extreme cases such as reported by Barbetti and Khangura (1999) in WA, where up to 70% of seedlings were killed by blackleg, yield is likely to be reduced. It was expected that the incidence and severity of stem infection would be more closely correlated to yield (Zhou *et al.* 1999; Barbetti and Khangura 2000). The reason for the low correlation between stem infection and yield in this study may be the limited data set obtained from only two trials, or even the use of the resistant cvs Hyola 60 and Pinnacle in these trials.

Stem canker was compared to yield separately for each cultivar, except for Hyola 60, on which stem canker did not occur. Yield of the blackleg resistant cvs Pinnacle, Scoop, Dunkeld and Mystic was not affected directly by stem canker, as shown by the poor correlation between stem canker and yield ($R^2 \leq 0.5$). Therefore, stem canker had little effect on the resistant cultivars. In the case of cv. Dunkeld, even though plants developed high levels of stem canker in some trials, yield was still not affected. Since only external symptoms of stem canker were assessed in this study, it is possible that there was relatively little internal stem canker in plants of cv. Dunkeld, hence, the restriction of water and nutrient transport would have been less, allowing normal ripening of the pods (Davies 1986, cited by West *et al.* 2001).

Stem canker was strongly correlated ($R^2 > 0.6$) with yield for the less resistant cvs Q2, Hyola 42, Karoo and Monty. Cvs Hyola 42 and Monty have higher potential yields in most situations than Karoo and Q2 and, hence, yield from these cultivars is more severely affected by stem canker. As the regression coefficients were significant ($P<0.05$), predictive models were formulated for these less resistant cultivars and allowed us to determine the extent to which stem canker was able to reduce yield. Again, these relationships need to be validated on a larger set of data.

The yield analyses, although preliminary, suggested that stem canker is the most important stage of the disease in terms of yield, and that yield becomes more sensitive to stem canker as resistance decreases.

A predictive model was formulated for incidence of stem canker using stepwise linear regression on a range of variables. The final model of best fit involved a number of variables

including; cultivar, cumulative rain pre-sowing, interaction between cultivar and cumulative rainfall pre-sowing, degree days during leaf growth and degree days throughout the season.

The coefficients of cumulative rainfall pre-sowing in the predictive equation (section 5.3.4.2) varied for each cultivar. Increased cumulative rainfall appeared to reduce the potential of stem canker on cvs Dunkeld, Karoo, Mystic and Q2, while it appeared to increase the likelihood of stem canker for cvs Hyola 42, Monty, Pinnacle and Scoop. Summer rainfall affects the maturation of pseudothecia and timing of ascospore release (Petrie 1994; Pérès *et al.* 1999), so we would expect that increased rainfall pre-sowing would increase the incidence of stem canker. This was indeed the case for cvs Hyola 42, Monty, Pinnacle and Scoop in the present study. However, prolonged rainfall may increase the rate of stubble decomposition or encourage early release of ascospores before the emergence of canola seedlings (West *et al.* 1999), potentially decreasing the incidence of stem canker. Due to factors such as stem width and height, cvs Dunkeld, Karoo, Mystic and Q2 may require less moisture than the other cultivars for stubble decomposition and pseudothecial maturation.

The variables of degree days during leaf growth and degree days throughout the season were negatively correlated with stem canker. Degree days are the sum of the mean temperatures of each day throughout a specified period. Therefore, as cumulative temperature increases, the incidence of stem canker decreases, since the optimal temperature for *L. maculans* growth is 20°C, above which the fungus is restricted (Biddulph *et al.* 1999).

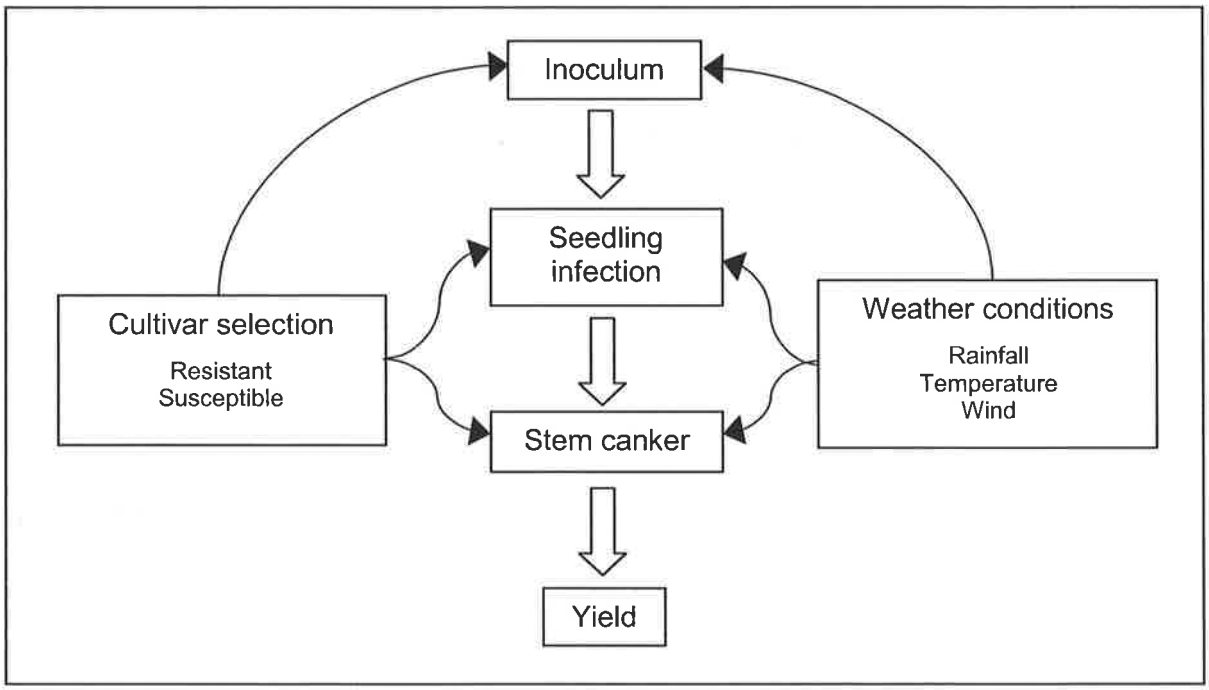
Analysis of the data obtained in this study allowed the identification of some environmental factors that appeared to have a significant effect on the incidence of blackleg stem canker in canola. However, due to the large amount of variability, these results should be viewed with some caution. By increasing the number of plants assessed for stem canker during flowering

or senescence to 20 plants per plot in at least 20 trials, a similar analysis could be conducted with more confidence in the results.

5.4.4 Summary

The use of field trials, spore trap plants and AWS in this study has been valuable for our understanding of the epidemiology of blackleg in canola in South Australian conditions. A schematic diagram was constructed from the results to illustrate the components of the disease cycle and the factors affecting them (Figure 5.13).

Figure 5.13: Schematic diagram of the blackleg disease model, showing the main components and factors affecting them.



Ascospore inoculum is produced from pseudothecia on canola stubble (section 2.4.2). Resistant cultivars have fewer stem cankers, which results in production of fewer pseudothecia and hence fewer ascospores than susceptible cultivars. Also, temperature and rainfall will influence stubble decomposition, maturation of pseudothecia and, finally, ascospore release (section 2.4.3). Wind speed and direction will determine how far the ascospores will be dispersed and where they will land.

Ascospore germination on and infection of cotyledons and leaves require optimal temperature and moisture conditions, as does the growth of the fungus into the stem.

Before the introduction of the highly resistant cv. Hyola 60, cultivar selection had little effect on seedling infection, but resistance at the cotyledon and leaf may become the basis for selection for resistance to blackleg stem canker in the future. Cultivar selection has played a major role in the management of stem canker since the 1980s, when Australian breeding programs began to focus on blackleg resistance (section 2.2.2).

Rainfall and temperature are the other factors that contribute to the incidence of stem canker throughout the season and, with better understanding of how they influence the disease, management strategies such as fungicide application, timing of sowing and stubble management could be improved.

Yield of canola seed is, therefore, dependent on a series of factors that occur throughout the growing season, as well as between seasons. With knowledge of these factors and their influence on disease, crops can be managed more effectively to prevent epidemics of blackleg.

CHAPTER 6

EPIDEMIOLOGY IN CONTROLLED ENVIRONMENTS

6.1 INTRODUCTION

Epidemics of blackleg vary from season to season and between regions. This suggests that the factors which affect the epidemiology of the disease will determine the extent of damage that will occur in a canola crop.

Inoculum occurs in two forms; ascospores, which are spread predominantly by wind, and pycnidiospores, which are spread mainly by rain-splash (Hall 1992). Wood and Barbetti (1977b) found that the concentration of either ascospores and pycnidiospores had little effect on disease incidence, whereas Hammond and Lewis (1987a) showed that raising pycnidiospore concentration could increase disease incidence and severity. This contradiction may be due to differences in methodology and highlights the need for further investigation.

Temperature and wetness period are important factors which affect the germination of ascospores and pycnidiospores, as well as the colonisation of the leaves and stems of canola plants by the fungus. Optimal conditions for germination of ascospores were shown to be 28°C with at least 4 h of continuous wetness, and for germination of pycnidiospores were shown to be 24°C with at least 16 h of continuous wetness (Hall 1992). Maximum leaf infection required 20°C and 48 h of leaf wetness (Biddulph *et al.* 1999; Toscano-Underwood *et al.* 2001), above and below which, the number of leaf lesions decreased. Stem cankers were most severe when temperatures ranged between 20°C and 24°C, and severity decreased outside of this range (Barbetti 1975; McGee and Petrie 1979; Gladders and Musa 1980).

The maturity of canola plants at the time of inoculation may affect the success of the infection and appears to vary between regions (West *et al.* 2001). McGee and Petrie (1979) found that inoculation after GS 2.6 led to development of stem canker too late to cause severe yield loss in south-eastern Australia. In the UK, Hammond and Lewis (1986a) found that, in the field, the most damaging cankers were formed from leaf infections which occurred before the onset of rapid stem extension and mainly from GS 2.3 – GS 2.10. However, the timing of ascospore release is such that in Australia it coincides with seedling emergence, but in the UK it occurs when plants are more mature (West *et al.* 2001). So, stem canker severity is likely to be dependent on the timing of ascospore release, which in turn, is dependent on temperature and rainfall, and ascospore release has been shown to vary between seasons and regions (section 5.4.2).

A range of canola cultivars with a wide variety of traits to suit different situations is commercially available to growers (Potter and Stanley 2002). The level of blackleg resistance in each cultivar has been determined in trials in field disease nurseries in all of the canola growing states of Australia, and ranked on a scale from 1 (most susceptible) to 9 (most resistant) (Marcroft 2001). A controlled environment experiment will allow us to determine whether the ranking is due solely to resistance or if environmental factors influence the response of the plant to infection.

Although the factors which affect blackleg infection have been studied extensively in the past, it is important to determine if the effect of these factors has changed with the development of new cultivars and more virulent isolates of *L. maculans*. Hence, a series of controlled environment experiments was carried out to investigate this possibility.

6.2 MATERIALS AND METHODS

6.2.1 Effect of inoculum concentration

Canola plants were inoculated with pycnidiospore and ascospore suspensions in order to determine the effect of spore type and concentration on the incidence of leaf and stem disease.

6.2.1.1 Preparation of pycnidiospore suspensions

Agar plugs of *L. maculans* isolate 66/97 were retrieved from storage (section 3.2.1) and cultured on ¼ PDA plates. The cultures were incubated at room temperature (approximately 22°C) for 2 weeks under blacklight (Hitachi 8W/BL350) and fluorescent light (Phillips 18W/W43) for 12 h each day. A pycnidiospore suspension was prepared as described in section 3.2.1.4 and adjusted to 1 x 10^7 pycnidiospores/ml by adding sterile RO water. The suspension was placed on a magnetic stirrer, and 25 ml were transferred to each of three McCartney bottles. Sterile RO water was added to the stock suspension, to achieve concentrations of 1 x 10^6, 10^5, 10^4 and 10^3 pycnidiospores/ml, and three McCartney bottles containing 25 ml were prepared for each concentration. Tween surfactant was added to each McCartney bottle at a concentration of 0.05%.

6.2.1.2 Preparation of ascospore suspensions

Several pieces of canola stubble bearing pseudothecia were collected from a commercial field during the season following a canola crop. The pieces of stubble were placed in a sealed plastic container lined with paper towel, moistened with RO water to promote high humidity, and left for 24 h at room temperature (approximately 22°C) to enable maturation of ascospores. Using similar methods to those of Barbetti (1975), pseudothecia were removed from stubble pieces with a scalpel and placed into a ceramic mortar with sterile RO water and then crushed with a pestle to release ascospores. Using a haemocytometer, the concentration was adjusted to 1 x 10^4 ascospores/ml by adding RO water and 25 ml transferred to each of

three McCartney bottles. A series of 5 x 10^2, 1 x 10^2, 5 x 10^1 and 1 x 10^1 ascospores/ml was produced by adding RO water and transferring 25 ml to each of three McCartney bottles as above. Tween surfactant was added to each McCartney bottle at a concentration of 0.05%.

6.2.1.3 Glasshouse experiments

Canola plants were inoculated with pycnidiospore or ascospore suspensions in the glasshouse as follows. Nine punnets (900 ml) were placed in each of 15 trays and filled with potting soil (section 3.1.2.1). The experiments were arranged as split plots with three replications, where seed of three cvs (Q2, Monty and Dunkeld) were sown into punnets (three seeds per punnet) so that each tray consisted of three punnets of each cultivar. After germination of seedlings, each punnet was thinned to two plants. When plants reached GS 2.3, each tray was sprayed evenly with 25 ml of spore suspension with one of the five concentrations. Two experiments were conducted so that pycnidiospores and ascospores could be tested separately. Trays were randomised and placed in humidity tents (240 x 95 x 150 cm, Figure 6.1A). These provided 80-100% relative humidity by automatically misting carbon-filtered tap water through fine sprayers for 1 s every 5 min (7 am - 7 pm) and 1 s every 10 min (7 pm - 7 am) with a Mist Master 11 electronic controller (Superior Controls Co. Inc.). After 4 days, the tents were opened to allow airflow, and misting continued, as above.

The number of leaf lesions on each plant was recorded 14 days after inoculation and then the number of plants with stem infection was recorded 37 days after inoculation.

6.2.2 Effect of temperature and wetness period

A series of experiments was conducted in an Environ Air controlled environment chamber (100 x 120 x 145 cm, with fan-forced refrigeration) to test the effect of temperature and wetness period on infection of canola by *L. maculans*. Light was provided by four metal

halide lights (Venture HIE 150W/C/U) and two cool white fluorescent tubes (VHO Sylvania 115W-F48T12) which were set at a 12 h photoperiod and controlled by a Theben (WF 64CA7) timer. Temperature was kept constant using an RKC REX-C100 temperature controller. Temperature and relative humidity were monitored using a data logger (Tinytag plus, Hasting Data Loggers).

The trial consisted of a series of eight experiments, each of which involved 28 pots (140 mm diameter) filled with potting soil (section 3.1.2.1). Each pot was sown with five seeds of cv. Hyola 42, maintained in a glasshouse as described in section 3.1.2.3, and the seedlings thinned to three per pot. The inoculum was prepared, as described in section 3.2.1.4, at 10^6 pycnidiospores/ml of the *L. maculans* isolate 66/97 (A-group). Once plants reached GS 2.3, the inoculum suspension was sprayed over the plants until run-off occurred (50 ml per replication). Pots were then placed in white trays inside the controlled environment chamber and tap water was poured into trays every few days to keep soil moist for the duration of the experiments (Figure 6.1B). Each experiment was arranged in a randomised complete block design with four replications and six wetness periods were produced by covering plants with plastic bags immediately after inoculation then removing them 3, 9, 24, 48, 72 and 96 h later. One pot in each replication was left uncovered as a control (0 h).

The entire trial was arranged as a split plot design with two replications. For each experiment, the temperature was set to one of the following four regimes 8/6°C day/night, 13/10°C, 18/15°C or 23/20°C, and maintained for 6 weeks. Each week the plants were rated for disease symptoms depending on the stage of the plant by either i) counting the number of leaf lesions (LLC), ii) rating leaf and petiole infection on a scale from 0-4 (LR), and iii) counting plants with stem infection (SIC).

Figure 6.1: A. Humidity tent, in a glasshouse, which kept relative humidity between 80-100% at all times by automatic misting using a Mist Master 11 electronic controller. B. Pots of canola plants in trays filled with water in the Environ Air controlled environment chamber.

6.2.3 Effect of maturity

An experiment was arranged as a randomised complete block design with three replications, to examine the effect of maturity on infection of canola by *L. maculans*. Punnets (550 ml) filled with potting soil (section 3.1.2.1) were sown with two seeds (cv. Hyola 42) and placed in three trays (12 punnets/tray). Trays were placed on a bench in a refrigerated glasshouse (17-22°C) and watered by hand once a day. This was repeated each week for 5 weeks and seedlings were thinned to one per punnet. Ten days after the fifth set of three trays was sown, all 15 trays of plants at five different growth stages (GS 1, GS 2.3, GS 2.5, GS 3.1 and GS 4.1) were inoculated by spraying with a suspension of pycnidiospores of *L. maculans* (10^6 pycnidiospores/ml, prepared as described in section 3.2.1.4) until run-off occurred. Trays were then placed into humidity tents (section 6.2.1.3) in a glasshouse (18-29°C) for 4 days and then the tents were opened and misting continued. The number of plants (of a total 12 per treatment) with leaf infection, stem infection and stem canker was counted 14, 21, 28, 36 and 49 days after inoculation.

6.2.4 Effect of cultivar

The effect of infection by *L. maculans* on a range of canola cvs (Hyola 60, Dunkeld, Mystic, Scoop, Oscar, Pinnacle, Monty, Karoo, Hyola 42 and Q2) that were used in field trials (section 5.2.1) was assessed in controlled conditions in the glasshouse. Trays, which contained nine punnets (900 ml) each filled with potting soil and sown with three seeds, contained one canola cultivar each. The experiment was arranged as a randomised complete block design with three replications. After seed had germinated, seedlings were thinned to leave one in each punnet, and when they reached GS 2.3 the entire plants were sprayed with a suspension of *L. maculans* isolate 66/97 (10^6 pycnidiospores/ml prepared as described in section 3.2.1.4) until run-off occurred. Trays were placed into closed humidity tents (section 6.2.1.3) for 4 days, after which the tents were opened and regular misting continued for the duration of the experiment. Fourteen days after inoculation, plants were rated for leaf infection, by counting the number of leaf lesions per tray. Stem infection was assessed 28 and 42 days after inoculation by counting the number of plants with stem infection. Fifty-five days after inoculation, plants were rated for stem canker (complete girdling).

6.3 RESULTS

6.3.1 Effect of inoculum concentration

The ANOVA for leaf lesion development after inoculation with pycnidiospores (Table 6.1) showed that the number of lesions per plant was significantly different ($P<0.05$) among the five concentrations of pycnidiospore inoculum. There was also a significant difference ($P<0.05$) between the number of leaf lesions on the three cultivars after inoculation with pycnidiospores.

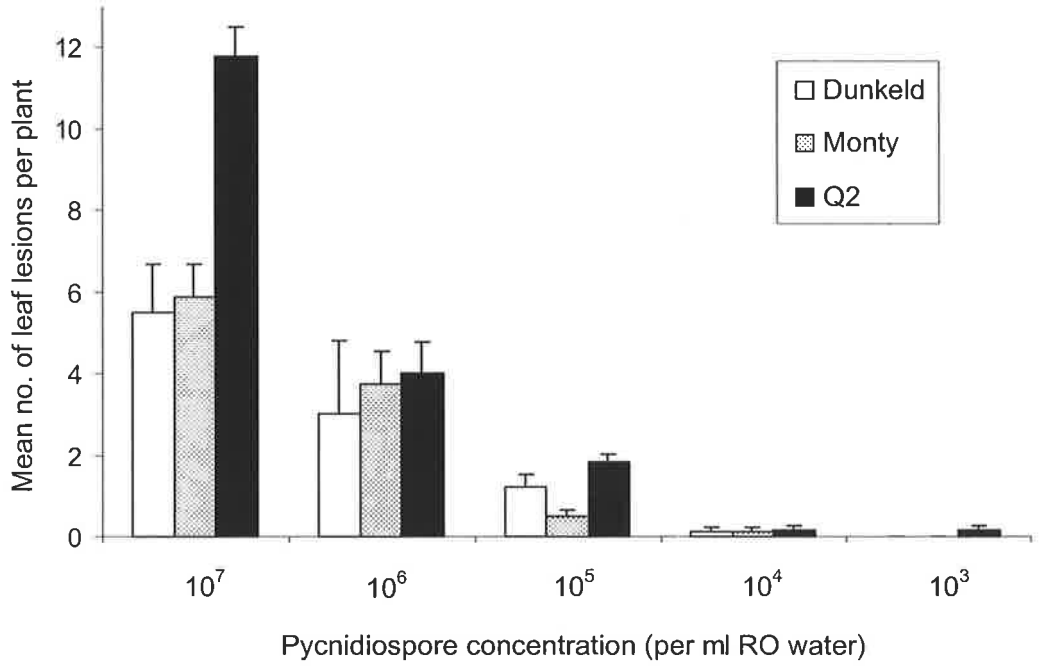
Figure 6.2 reveals that very few leaf lesions developed at the two lowest concentrations (10^3 and 10^4 pycnidiospores/ml RO water) and then for each ten-fold increase in concentration, the mean number of leaf lesions on the cultivars, combined, increased significantly up to 10^7

pycnidiospores/ml. At 10^7 pycnidiospores/ml, there was a significant difference between cv. Q2 and cvs Monty and Dunkeld, with approximately 12 and six leaf lesions per plant, respectively, yet there was no difference between cultivars at 10^6 pycnidiopores/ml. The development of more leaf infection on cv. Q2 than on the other two cultivars at the highest concentration only is the reason for the significant interaction ($P<0.05$) between pycnidiospore concentration and cultivar shown in Table 6.1.

Table 6.1: Analysis of variance table for number of leaf lesions per plant on three canola cultivars (Dunkeld, Monty and Q2), 14 days after inoculation with one of five pycnidiospore suspensions (10^7, 10^6, 10^5, 10^4 and 10^3 pycnidospores/ml RO water) of *L. maculans* isolate 66/97.

Source	df	ss	ms	f	p
Replication (A)	2	1.97	0.99	0.66	0.523
Concentration (B)	4	375.68	93.92	63.24	0.000
Cultivar (C)	2	25.11	12.56	8.46	0.001
B*C	8	53.46	6.68	4.50	0.001
A*B*C	28	41.59	1.49		
Total	44	497.81			

Figure 6.2: Effect of pycnidiospore concentration and canola cultivar on the mean number of leaf lesions per plant, 14 days after inoculation with *L. maculans* isolate 66/97, with three replications per treatment. Bars represent standard error of the mean.



The ANOVA for stem canker development after inoculation with pycnidiospores (Table 6.2) revealed a significant difference ($P<0.05$) between pycnidiospore concentrations and between cultivars. At the two lowest concentrations (10^3 and 10^4 pycnidiospores/ml), stem canker developed on fewer than 20% of the plants, and there was no infection on cv. Dunkeld (Figure 6.3). When the concentration was increased to 10^5 pycnidiospores/ml, the incidence of stem canker increased significantly ($P<0.05$) to approximately 50% for cvs Q2 and Monty but only to 10% for cv. Dunkeld. A further ten-fold increase in concentration to 10^6 pycnidiospores/ml significantly increased the incidence of stem canker to between 70% and 80% for cvs Q2 and Monty, but the incidence of stem canker of cv. Dunkeld remained at 10%. Increasing the concentration to 10^7 pycnidiospores/ml appeared to have no effect on cvs Q2 and Monty, but increased the incidence of stem canker on cv. Dunkeld to over 20%.

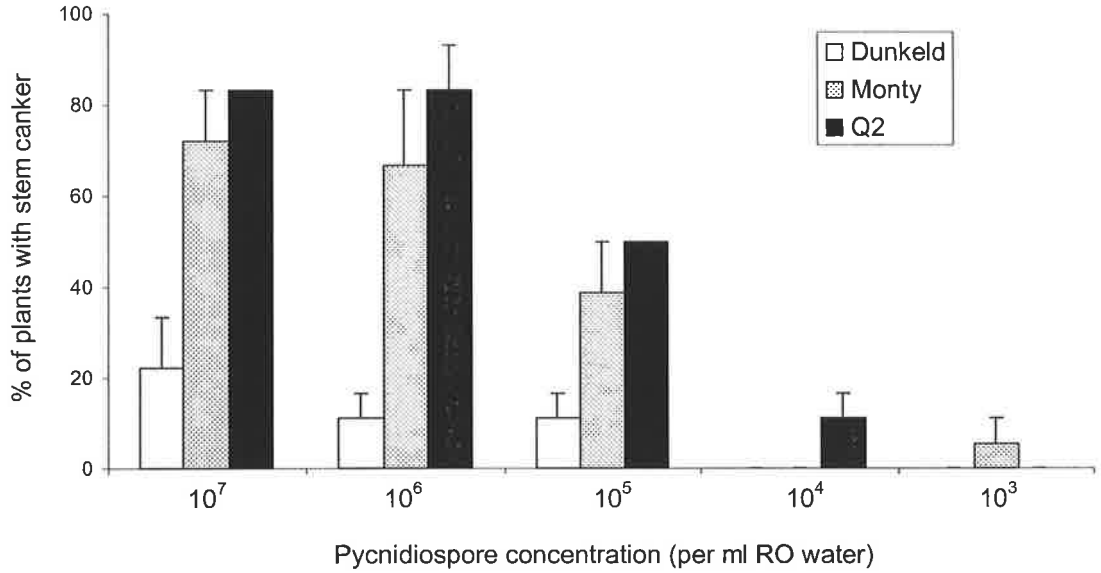
The significant interaction ($P<0.05$) between pycnidiospore concentration and cultivar was due to the large increase in stem canker on cvs Q2 and Monty with increased pycnidiospore concentration compared to the small change in stem canker incidence on cv. Dunkeld.

In the experiment in which canola plants were inoculated with ascospores, very few leaf lesions developed and there was no obvious difference between cultivars or concentrations. Furthermore, no stem canker was observed on any of the plants. Therefore results are not shown.

Table 6.2: Analysis of variance table for number of plants with stem canker (of 6) on three commercial cultivars (Dunkeld, Monty and Q2), 37 days after inoculation with one of five pycnidiospore suspensions (10^7, 10^6, 10^5, 10^4 and 10^3 pycnidospores/ml RO water) of *L. maculans* isolate 66/97.

Source	df	ss	ms	f	p
Replication (A)	2	3.51	1.76	3.24	0.054
Concentration (B)	4	94.36	23.59	43.58	0.000
Cultivar (C)	2	39.51	19.76	36.50	0.000
B*C	8	24.04	3.01	5.55	0.000
A*B*C	28	15.16	0.54		
TOTAL	44	176.58			

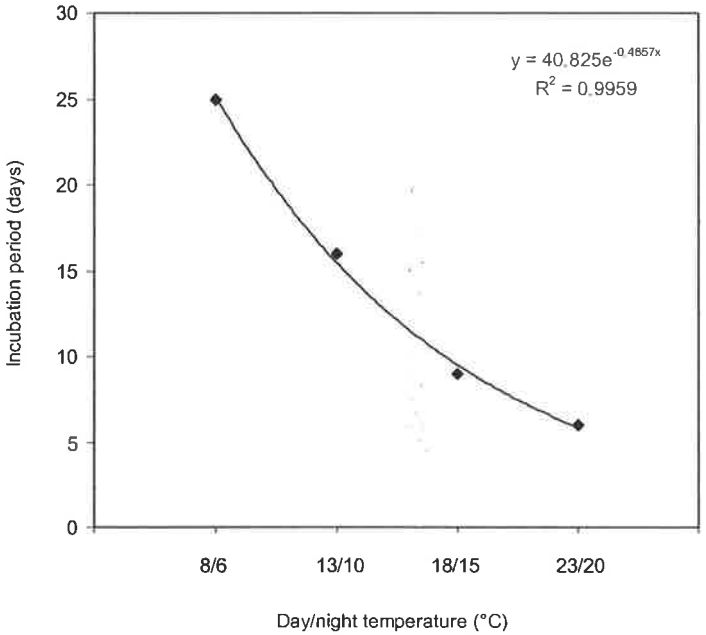
Figure 6.3: Effect of pycnidiospore concentration and canola cultivar on the incidence of stem canker, 37 days after inoculation with *L. maculans* isolate 66/97, with three replications per treatment. Bars represent standard error of the mean.



6.3.2 Effect of temperature and wetness period

Leaf lesions were first detected on canola plants (cv. Hyola 42) 25 days after inoculation at the day/night temperature of 8/6°C, 16 days at 13/10°C, 9 days at 18/15°C and 6 days at 23/20°C. Figure 6.4 reveals that the regression between latent period and temperature was exponential. Plants were checked every day until the first leaf lesions were observed, and disease was assessed at 7 day intervals after inoculation. Figure 6.4 shows the number of leaf lesions which had developed by the first weekly assessment time after lesions were first observed at each of the four temperature regimes. As the day/night temperature increased from 8/6°C to 23/20°C, so too did the number of leaf lesions. At the higher temperatures (18/15°C and 23/20°C), significantly fewer ($P<0.05$) leaf lesions occurred at wetness periods of between 0 h and 48 h than wetness periods of 72 h and 96 h. At 8/6°C and 13/10°C, the number of leaf lesions remained below 10, and there was no significant difference ($P>0.05$) between wetness periods.

Figure 6.4: Regression analysis of latent period (days from inoculation with pycnidiospores of *L. maculans* isolate 66/97 to appearance of first leaf lesions) on canola cv. Hyola 42 and temperature (day/night) with two replications of each treatment.



The ANOVA for stem infection (Table 6.3) shows that there was a significant difference ($P<0.05$) among both temperatures and wetness periods for the number of plants of the cv. Hyola 42 with stem infection, but there was no significant ($P>0.05$) interaction between these factors. Figure 6.6 shows the effect of temperature and wetness period on the incidence of stem infection on plants 42 days after inoculation. At 8/6°C, no stem infection developed on the plants at any wetness period, nor was stem infection observed on plants incubated at 13/10°C, except for the 96-h wetness period, after which 10% of the plants developed stem infection. At 18/15°C, as the wetness period increased from 0 h to 96 h, the incidence of stem infection increased from 10% to 60%, but at wetness periods 24 h and 48 h, only between 15% and 20% of plants developed stem infection. At 23/20°C, as wetness period increased from 0 h to 48 h, the incidence of stem infection increased from 20% to over 80% and then plateaued between 48 h and 96 h. Figure 6.7 shows plants at the end of an experiment which was conducted at 23/20°C.

Figure 6.5: Effect of temperature (day/night) and wetness period on the number of leaf lesions which developed on 12 canola plants (cv. Hyola 42) and latent period after inoculation with pycnidiospores of *L. maculans* isolate 66/97 with two replications of each treatment. Bars represent standard error of the mean.

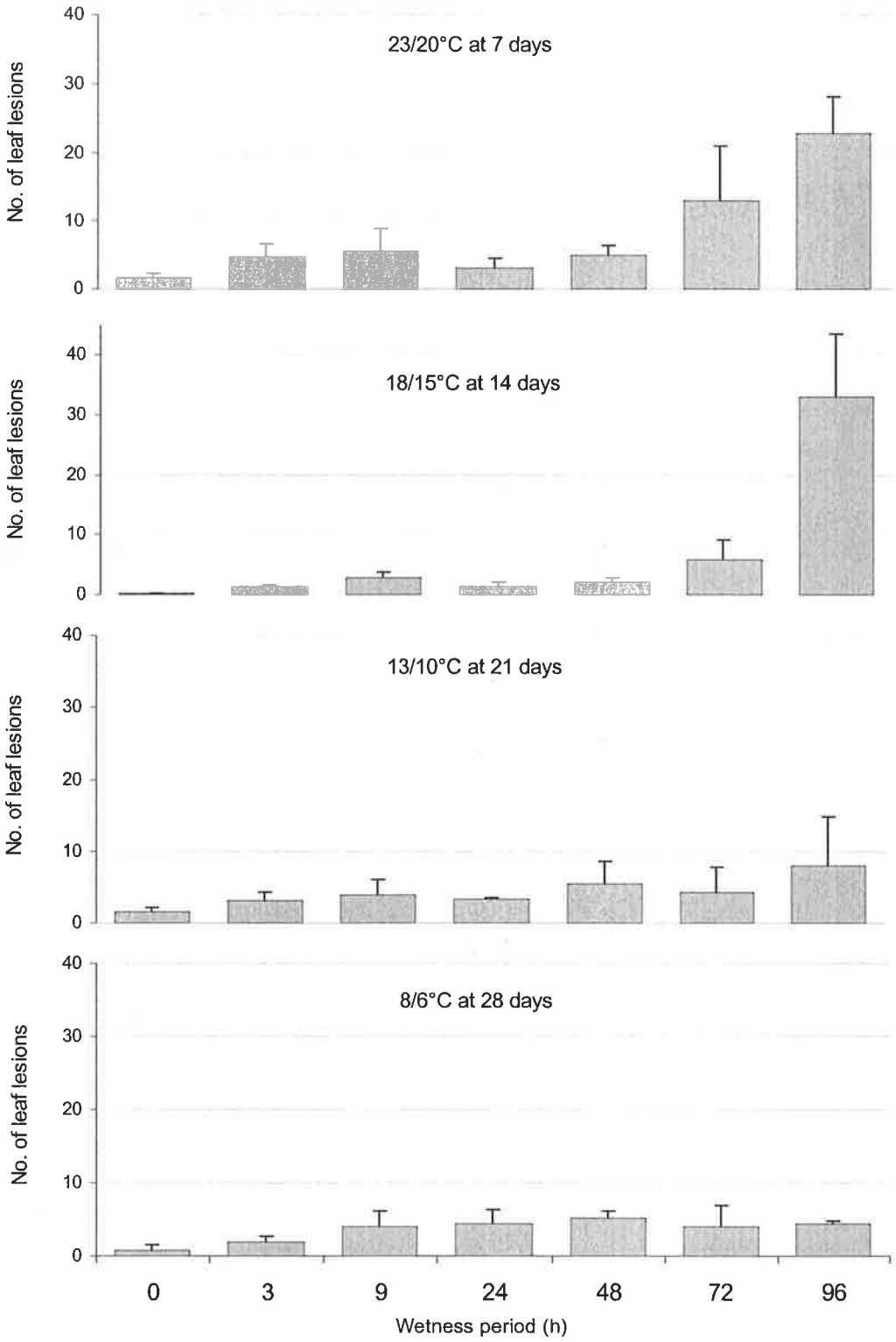
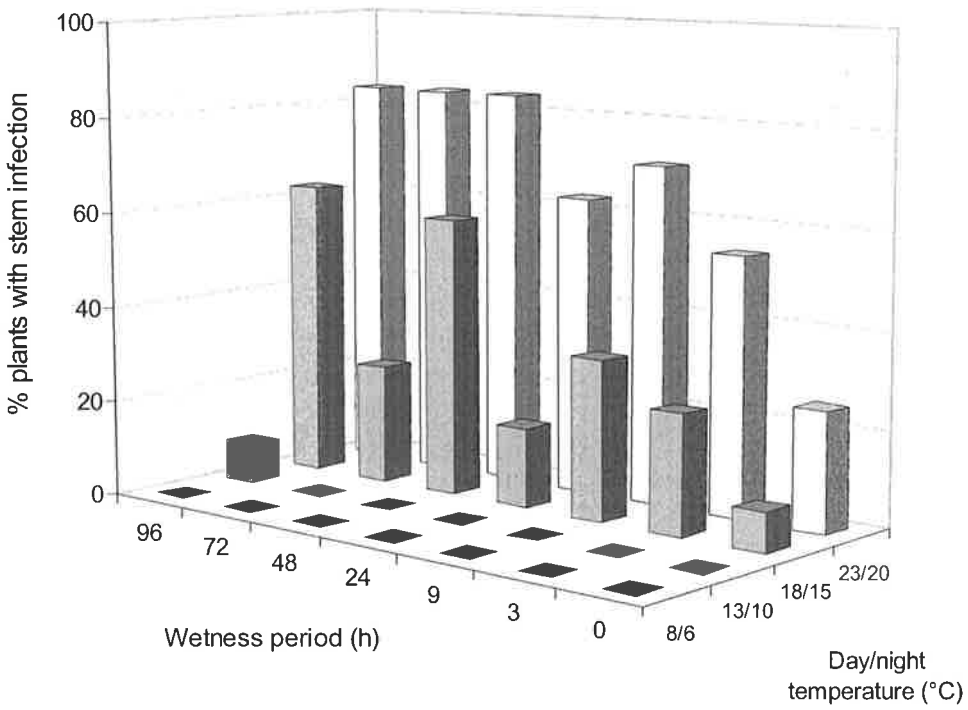


Table 6.3: Analysis of variance table for temperature and wetness period on the number of plants with stem infection (of 12), 42 days after inoculation with pycnidiospores of *L. maculans* isolate 66/97.

Source	df	ss	ms	f	p
Replication (A)	1	1.78	1.786	0.63	0.433
Temperature (B)	3	592.43	197.48	69.96	0.000
Wetness period (C)	6	72.50	12.08	4.28	0.004
B*C	18	81.07	4.50	1.60	0.133
A*B*C	27	76.21	2.82		
TOTAL	55	824.00			

Figure 6.6: Effect of temperature(day/night) and wetness period on the percentage of plants (cv. Hyola 42) with stem infection, 42 days after inoculation with pycnidiospores of *L. maculans* isolate 66/97 with two replications per treatment.



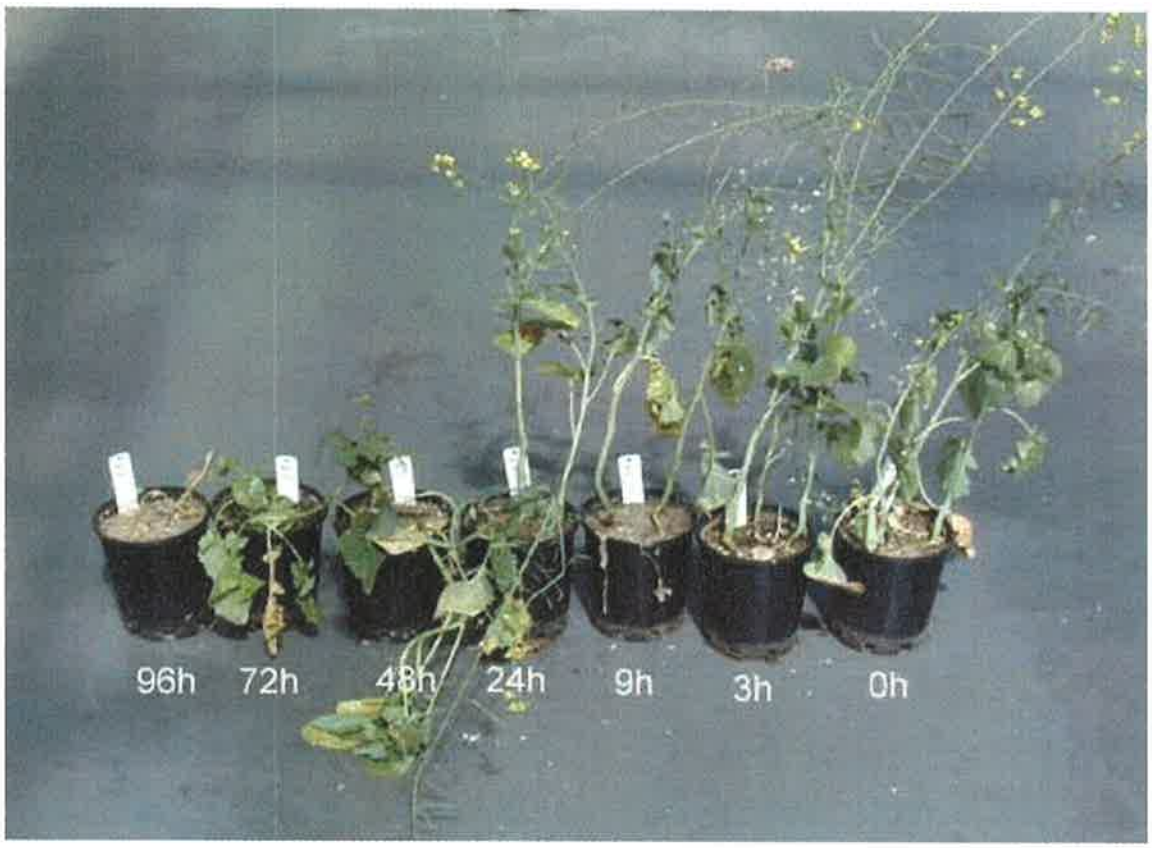


Figure 6.7: Canola plants (cv. Hyola 42) which were subjected to wetness periods from 0 to 96 h by covering with plastic bags and day/night temperatures of 23/20°C inside a controlled environment cabinet for 6 weeks after inoculation with pycnidiospores of *L. maculans* isolate 66/97.

6.3.3 Effect of maturity

Table 6.4 shows the effect of plant maturity at inoculation on the mean number of canola plants (cv. Hyola 42) with leaf and stem infection at three different times after inoculation. Fourteen days after inoculation, there was no significant difference ($P>0.05$) in the number of plants with leaf infection among the five different growth stages. Plants inoculated at GS 1 to GS 2.5 developed significantly more ($P<0.05$) stem infection than plants inoculated at GS 3.1 to GS 4.1, 28 days after inoculation. The final assessment at 49 days after inoculation revealed that plants inoculated at GS 1 and GS 2.3 had more than 80% incidence of stem canker, significantly more than plants inoculated at GS 2.5, which had 25% incidence of stem canker (Figure 6.8). The incidence of stem canker on plants inoculated at GS 3.1 and GS 4.1 was 2% and 0% respectively, significantly less ($P<0.05$) than that for plants inoculated at the other growth stages.

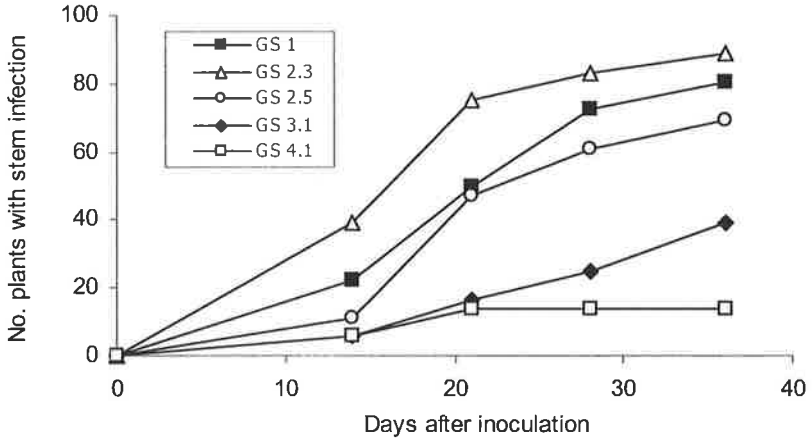
Table 6.4: Effect of maturity of canola plants at inoculation with *L. maculans* on number of plants (of 12) with leaf and stem infection 14, 28 and 49 days after inoculation with *L. maculans* isolate 66/97 with three replications per treatment. Values within a column with the same superscript are not significantly different ($LSD_{0.05}$).

	14 days after inoculation		28 days after inoculation		49 days after inoculation	
Maturity at inoculation	Maturity at assessment	Leaf infection	Maturity at assessment	Stem infection	Maturity at assessment	Stem canker
GS 1	GS 2.3	8.7[a]	GS 3.2	8.7[a]	GS 4.4	10.0[a]
GS 2.3	GS 3.1	12.0[a]	GS 4.1	10.0[a]	GS 5.1	10.3[a]
GS 2.5	GS 4.1	12.0[a]	GS 4.3	7.3[a]	GS 5.2	4.0[b]
GS 3.1	GS 4.3	10.3[a]	GS 5.1	3.0[b]	GS 5.4	0.3[c]
GS 4.1	GS 4.4	11.7[a]	GS 5.2	1.7[b]	GS 5.4	0.0[c]

Figure 6.8: Canola plants (cv. Hyola 42), 49 days after inoculation with pycnidiospores of *L. maculans* isolate 66/97 at five growth stages (GS 1 to GS 4.1) and incubation in the glasshouse.

Figure 6.9 shows the effect of maturity at inoculation on the incidence of stem infection over time. During the first 21 days after inoculation, stem infection occurred more rapidly on plants inoculated at GS 1, GS 2.3 and GS 2.5 than on plants inoculated at GS 3.1 and GS 4.1. Between 21 and 36 days after inoculation, the rate of stem infection increase was reduced for plants inoculated at all growth stages and, for plants inoculated at GS 4.1, the incidence was unchanged during the last 2 weeks of the experiment.

Figure 6.9: Effect of maturity of canola plants (cv. Hyola 42) at inoculation on the incidence of stem infection over time after inoculation with *L. maculans* isolate 66/97 in the glasshouse, with three replications per treatment. LSD (14 days) = 20.8; LSD (21 days) = 29.2; LSD (28 days) = 28.3; LSD (36 days) = 28.8.



6.3.4 Effect of cultivar

Table 6.5 shows the extent of infection on the leaves and stems of canola cultivars at different times after inoculation with *L. maculans*. Fourteen days after inoculation, there was significantly more ($P<0.05$) leaf infection on cv. Scoop than on cvs Dunkeld, Mystic, Pinnacle and Karoo. Cvs Hyola 42 and Q2 had developed significantly more ($P<0.05$) stem infection than all other cultivars except cvs Monty and Karoo at both 28 and 42 days after inoculation. Few plants of cvs Mystic, Pinnacle, Dunkeld and Hyola 60 showed symptoms of stem infection at 28 and 42 days after inoculation, and showed significantly less ($P<0.05$) stem infection than cvs Hyola 42 and Q2. Fifty-five days after inoculation, significantly more ($P<0.05$) plants of cvs Q2 and Hyola 42 had stem canker than all other cultivars, and cvs Mystic, Scoop, Dunkeld, Pinnacle and Hyola 60 had significantly less ($P<0.05$) stem canker than cvs Q2, Hyola 42 and Karoo.

The incidence of stem infection increased at different rates for each cultivar (Figure 6.10). Fifty-five days after inoculation, the incidence of stem canker on the nine cultivars tested

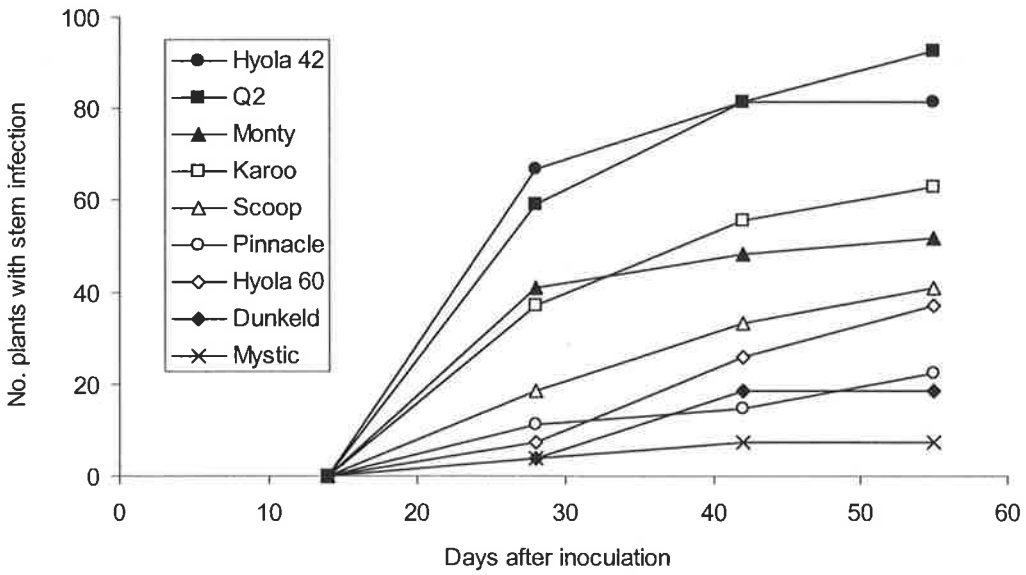
ranged from 5% to 90%. The greatest incidence of stem infection occurred on the cvs Hyola 42 and Q2 and the lowest incidence of stem infection occurred on the cvs Mystic, Dunkeld and Pinnacle.

Table 6.5: Effect of canola cultivar on; leaf infection 14 days after inoculation, stem infection 28 and 42 days after inoculation and stem canker 55 days after inoculation with *L. maculans* isolate 66/97 with three replications per treatment. Values within a column with the same superscript are not significantly different ($LSD_{0.05}$).

14 days after inoculation		28 days after inoculation		42 days after inoculation		55 days after inoculation	
Cultivar	Leaf infection#	Cultivar	Stem infection*	Cultivar	Stem infection*	Cultivar	Stem canker*
Scoop	45.7^{a}	Hyola 42	6.0^{a}	Hyola42	7.3^{a}	Q2	8.3^{a}
Hyola 42	38.0^{ab}	Q2	5.3^{ab}	Q2	7.3^{a}	Hyola42	7.3^{a}
Monty	36.3^{ab}	Monty	3.7^{bc}	Karoo	5.0^{ab}	Karoo	3.3^{b}
Q2	34.3^{ab}	Karoo	3.3^{bc}	Monty	4.3^{bc}	Monty	1.7^{bc}
Hyola 60	25.7^{ab}	Scoop	1.7^{cd}	Scoop	3.0^{bcd}	Hyola60	1.3^{c}
Dunkeld	24.7^{b}	Pinnacle	1.0^{d}	Hyola60	2.3^{bcd}	Pinnacle	1.3^{c}
Mystic	22.0^{b}	Hyola 60	0.7^{d}	Dunkeld	1.7^{cd}	Dunkeld	1.0^{c}
Pinnacle	21.0^{b}	Dunkeld	0.3^{d}	Pinnacle	1.3^{d}	Scoop	0.3^{c}
Karoo	20.0^{b}	Mystic	0.3^{d}	Mystic	0.7^{d}	Mystic	0.3^{c}

mean number of leaf lesions per tray (9 plants)
* mean number of plants with stem infection or canker (per tray of 9 plants)

Figure 6.10: Effect of canola cultivar on the incidence of stem infection over time after inoculation with pycnidiospores of *L. maculans* isolate 66/97 in the glasshouse, with three replications per treatment.



6.4 DISCUSSION

6.4.1 Effect of inoculum concentration

The concentration of pycnidiospores in the inoculum had a significant effect on both leaf and stem infection, which confirmed findings by Hammond and Lewis (1987a). However, there was no assessment of the viability of spores at inoculation, so the concentrations of viable pycnidiospores were unknown. The highest inoculum concentration used in this study (10^7 pycnidiospores/ml) resulted in the most leaf infection, and hence at least 10^7 pycnidiospores/ml should be used for maximum leaf infection, but more studies would be necessary to determine if concentrations above 10^7 pycnidiospores/ml will increase leaf infection further. The significant interaction between cultivar and inoculum concentration was due to the greater difference in leaf infection on plants of the susceptible cv. Q2 and the resistant cultivars at the highest inoculum concentration compared to that at lower concentrations. The interaction highlights the importance of using resistant cultivars in growing regions where inoculum potential is likely to be high, such as high rainfall areas with a high density of canola crops.

Raising the concentration of pycnidiospores in the inoculum of isolate 66/97 from 10^3 to 10^6 pycnidiospores/ml caused the incidence of stem canker to increase on the susceptible cvs Monty and Q2, but no more stem canker occurred when the concentration was raised again to 10^7 pycnidiospores/ml. Hence, the maximum incidence of stem canker was produced using 10^6 pycnidiospores/ml, but assessment of the viability of spores at inoculation would be needed to confirm this. The resistant and susceptible cultivars could be distinguished on the basis of the incidence of stem canker resulting from inoculation with pycnidiospores of isolate 66/97. So in growing regions in which there is little inoculum potential, it would be possible to grow less resistant cultivars, which may have other advantages such as early maturity or

increased yield, but as inoculum potential increases it would become more important that resistant cultivars be used to avoid yield loss due to blackleg.

Ascospore inoculum produced very little disease in this study, possibly due to the methods used to obtain the ascospores from pseudothecia on canola stubble (section 6.2.1.2). Firstly, ascospores may not have been fully mature when they were removed from the pseudothecia, and hence were not able to germinate on the canola plants. Secondly, the process of crushing pseudothecia with a mortar and pestle could have damaged the ascospores. These problems could have been avoided by assessing spore viability. Although ascospores have been shown to be more efficient at inducing disease than pycnidiospores (Wood and Barbetti 1977b), ascospores were unreliable and difficult to prepare in this study. Furthermore, it is not possible to compare isolates of *L. maculans* when ascospores are used as inoculum, as it is with pycnidiospores which can be cultured from single spores.

Although the concentration of ascospore and pycnidiospore inoculum in the field is not known, it is likely to be variable and can be reduced through the use of resistant cultivars. As pycnidiospore inoculum resulted in severe infection and was easy to produce *in vitro*, it was used in most experiments in this project.

6.4.2 Effect of temperature and wetness period

There was an exponential regression between latent period and temperature (Figure 6.4). Biddulph *et al.* (1999) found that leaf lesions occurred 5 days after inoculation at 20°C and after 14 days at 8°C. In this experiment, the predicted latent period at 20°C was similar, but was 25 days at 8°C. This difference is most likely due to two reasons, 1) the greater blackleg susceptibility of cv. Nickel, used by Biddulph *et al.* (1999), compared to cv. Hyola 42, which

was used in this study and 2) the night temperature dropped to 6°C in this experiment whereas it remained constantly at 8°C in the past study.

The optimal day/night temperature and wetness period regime for leaf infection in the present study was 18/15°C and 96 h. In comparison, Biddulph *et al.* (1999) showed that the optimal conditions were 20°C and a 48 h wetness period and Toscano-Underwood *et al.* (2001) reported optimal conditions of 15 to 20°C and a 48 h wetness period. The optimal temperature in this study corresponded with that found in past studies, but wetness period did not. As 96 h was the longest wetness period tested in this experiment, it is not known if the amount of infection would have increased further with a longer wetness period. The reason for the difference in optimal wetness periods could be due to the timing of disease assessment, which differed for each study. A small amount of leaf infection was detected on plants subjected to no wetness period (0 h) and 3 h wetness period. This was expected, as the controlled environment chamber ranged between 60% and 100% relative humidity, depending on the amount of water present in the trays underneath the pots. As plants were sprayed with inoculum in water, this could have contributed to a short wetness period for the control as well. As plants were watered immediately after inoculation, humidity would have ranged between 80% and 100% over the following 24 to 48 h, especially at night, when the lights were off. In retrospect, plants should have been watered at least 24 h before inoculation.

The optimal conditions for development of stem canker were 23/20°C with a 48-72 h wetness period. Thus, at least 48 h of leaf wetness was required post-inoculation for the maximum number of plants to develop stem canker. Previous studies revealed that the optimal temperature for stem canker to occur ranged between 20°C and 24°C (Barbetti 1975; McGee and Petrie 1979), which corresponded with the present study. At 18/15°C, there was only a small incidence of stem infection at 24 h and 72 h, compared to 9 h and 48 h, which was most

likely due to experimental error, such as the plastic bags being sealed and maintaining 100% relative humidity. No stem canker occurred at 8/6°C even though previous studies have shown that cankers develop, albeit slowly, between 4°C and 8°C. Even at 13/10°C, only a small number of plants subjected to the 96 h wetness period developed stem canker. Therefore, one of the reasons for the greater prevalence and severity of stem canker in Australia than in Europe and Canada is that average temperatures are greater in the former (West *et al.* 2001, Purwantara *et al.* 2002).

6.4.3 Effect of maturity

The maturity of Hyola 42 plants at the time of inoculation had no effect on the incidence of leaf infection. There have been several reports that younger leaves are more susceptible than older leaves to infection (Hammond and Lewis 1987a; McGee and Petrie 1979; Poisson and Pérès 1999) whereas Badawy *et al.* (1991) reported that older leaves were more susceptible. These contradictions are considered most likely to be due to the different assessment methods used in each study.

However, it is important to determine the effect of plant maturity at time of inoculation on the incidence of stem canker, since this factor has an impact on yield. Plants inoculated at the elongation stage (GS 3.1) or later, had virtually no stem canker 49 days later. Some infection was evident further up the stem of these plants, as spread of the fungus from new leaves of extended plants could only cause upper stem lesions (West *et al.* 1999). The incidence of stem canker on plants inoculated at GS 2.5 was about 30%, but on plants inoculated at GS 2.3 or earlier the incidence of stem canker was more than 80%. Therefore, the younger the plant at inoculation, the more vulnerable it was to stem canker infection. McGee and Emmett (1977) found that stems were most susceptible up until the two-leaf stage (GS 2.2), and

subsequently, showed that infection must occur before the six-leaf stage for severe stem cankers to develop (McGee and Petrie 1979).

It appears that plants inoculated when young are more likely to develop stem canker than those inoculated when more mature, and this is due to the physical distance from the leaf to the base of the stem. Therefore, younger plants are not less resistant, rather, they are physically more vulnerable to stem infection.

6.4.4 Effect of cultivar

There was very little effect of cultivar on leaf infection, which was to be expected as canola breeding programs select only for blackleg stem resistance, and leaf and stem resistance are not genetically linked (Roy 1978a). The glasshouse experiment confirmed field results showing that most cultivars did not have resistance to leaf infection (section 5.3.1), although cv. Hyola 60 developed significantly less leaf infection than all other cultivars in the field. This difference may be related to the large number of pycnidiospores applied in the glasshouse, whereas the plants would have been exposed to relatively few spores in the field.

As plants matured, the incidence of stem infection increased more for some cultivars than for others. By the last assessment, 55 days after inoculation, there were significant differences among cultivars in terms of incidence of stem canker. The most susceptible cultivars were Q2 and Hyola 42. In field trials, these two cultivars were also the most susceptible. Interestingly, cv. Hyola 60, which was extremely resistant in the field, was not significantly different to the cvs Mystic, Scoop, Dunkeld, Pinnacle and Monty in the glasshouse experiment. Furthermore, cv. Pinnacle, which was significantly more resistant than all other cultivars (except Hyola 60) in the field, also was not significantly different than some of the cultivars under controlled conditions. So, it appears that all cultivars other than Hyola 60 and Pinnacle exhibited better

resistance to blackleg in the glasshouse than in the field. The reason for this difference may be that the controlled environment provided optimal climatic conditions and nutrition, free from any other diseases or insects, compared to the field. Therefore, cvs Hyola 60 and Pinnacle may be better suited agronomically to the environments of the two field trials than the other cultivars tested and so their resistance to blackleg was more effective in the field than the other cultivars.

The ranking of blackleg resistance of the canola cultivars in a controlled environment differed from both field trials in South Australia and national Australian resistance rankings (Potter and Stanley 2002). This highlights the importance of cultivar selection for growers in different areas and may mean that blackleg resistance ratings should be determined on a regional basis in south-eastern Australia. The different environmental conditions in Western Australia have compelled the industry in that state to produce independent blackleg resistance ratings (Khangura *et al.* 2002).

6.4.5 Summary

Controlled environment experiments have revealed that inoculum concentration, temperature, wetness period, plant maturity and cultivar all affect the epidemiology of blackleg in canola. They have allowed us to determine how each factor affects disease progression. Together with results from the field trials, they give us a clearer picture of how the pathogen reacts to outside influences and provide a valuable source of information which can be used in developing new management strategies or fine tuning existing ones, to minimise yield loss due to blackleg. For example, foliar fungicides for blackleg, which are uneconomical and no more effective than Impact® in-furrow treatments (R. Khangura, personal communication 2001), may be appropriate if used only when plants are at their most vulnerable stage (GS 1 – GS 2.3) and when temperature and wetness periods are most conducive to infection.

CHAPTER 7

VALIDATION OF A DNA-BASED ASSAY FOR *L. MACULANS*

7.1 INTRODUCTION

Isolates of *L. maculans* collected in a previous study (Sosnowski 1999) were used in the development of DNA primers specific to *L. maculans* by CSIRO, Division of Entomology in Canberra. The *L. maculans* specific DNA primers are based on the sequence of the ITS region and can be used to differentiate between A and B-group strains (Sosnowski *et al.* 2001b). A DNA-based assay, involving the amplification of target DNA using the *L. maculans* specific primers in a PCR, was developed by the Root Disease Testing Service (RDTS), SARDI. The next stage in the development of this DNA-based assay was to assess its ability to quantify *L. maculans* DNA in stubble and soil samples.

DNA-based assays have been developed for a number of other plant pathogens such as *Phytophthora cinnamomi* in avocado roots (Judelson and Messenger-Routh 1996), a range of *Pythium* species (Klassen *et al.* 1996), *Gaeumannomyces graminis* var. *tritici* (*Ggt*) in soil (Herdina and Roget 2000) and *Uncinula necator* in grapes (Evans *et al.* 1996). Herdina and Roget (2000) validated a *Ggt*-specific DNA probe for use in a slot-blot hybridisation assay by correlating results with a soil bioassay and then determined the techniques necessary for obtaining representative samples in a field. Siwek and Ophel-Keller (1997) calibrated a DNA-based soil assay for the detection of *Rhizoctonia solani* AG-8 by correlating disease severity in a plant bioassay with the amount of *R. solani* DNA in the soil. A DNA test offered by the RDTS, SARDI for root lesion nematode, *Pratylenchus thornei*, was calibrated by correlating results against manual counts of nematodes in field soils and with soils containing known amounts of added inoculum (Ophel-Keller *et al.* 1999).

In this chapter, the steps taken in the development of a selective medium for the enumeration of *L. maculans* are described. A method for determining the most probable number (MPN) of *L. maculans* spores or propagules in stubble was adapted from a method used to quantify *Rhizobium* spp. in soil (Somasegaran and Hoben 1985). This method has recently been adapted for determining the MPN of colony forming units (CFU) of *Mycosphaerella pinodes* and *Phoma medicaginis* var. *pinodella* (blackspot of field peas) in soil (Davidson *et al.* 1999). A plant bioassay technique was also developed in the present study. Correlations were made between these two approaches and a DNA-based assay using the *L. maculans* specific primers. In addition, requirements for collecting representative soil samples from a field were determined.

7.2 MATERIALS AND METHODS

7.2.1 Development of selective medium for *L. maculans*

Experiment 1

Bretag (1991) and Davidson *et al.* (1999) enumerated *M. pinodes* and *P. medicaginis* var. *pinodella* in soil using Coon's medium, which was based on the formula described by Booth *et al.* (1971), with anti-microbial agents as in Nelson *et al.* (1983). An experiment was conducted to assess the growth of *L. maculans* on this medium.

Modified Coon's medium

RO water	1000 ml
Bacto agar (Difco)	20 g
Maltose (Sigma)	4 g
Potassium dihydrogen phosphate (BDH)	2.7 g
Potassium nitrate (BDH)	2 g
Magnesium sulphate (BDH)	1.2 g
1% Erythromycin (Sigma)	10 ml

Pentachloronitrobenzene – PCNB (Sigma)	1 g
Oxgall-bile (Sigma)	0.5 g
Sodium tetraborate (BDH)	1 g

The agar, maltose, potassium dihydrogen phosphate, potassium nitrate and magnesium sulphate and RO water were autoclaved at 121°C for 20 min. The anti-microbial agents; erythromycin (10 ml of 1 g/100 ml sterile RO water), PCNB, oxgall-bile and sodium tetraborate, were added aseptically to the molten medium, cooled to approx 50°C. The agar medium was dispensed into 9-cm diameter Petri dishes, approximately 15 ml per plate, and allowed to solidify.

L. maculans isolates 66/97 (A-group) and 213/97 (B-group) were grown from stored plugs (section 3.2.1) on modified Coon's medium (one plug per plate) at room temperature (approximately 22°C) under blacklight (Hitachi 8W/BL350) and fluorescent light (Phillips 18W/W43) for 12 h each day. Cultures were assessed 8 days later. The same isolates were also grown on modified Coon's medium without anti-microbial agents and assessed 7 and 14 days later by measuring the diameter of the colony on each plate and recording the presence or absence of pycnidia.

Three *L. maculans* isolates (66/97, 213/97 and 119/97) were then cultured from stored plugs (section 3.2.1) on modified Coon's medium containing either oxgall-bile, PCNB, erythromycin or sodium tetraborate separately, and assessed 13 days later as described above.

Experiment 2

As *L. maculans* grew well on ¼ PDA (section 3.2.1), and more rapidly than on modified Coon's medium (Experiment 1), an experiment was conducted to assess growth on ¼ PDA

amended with erythromycin and Rose Bengal, a selectively anti-fungal agent (King *et al.* 1979; Dhingra and Sinclair 1995), with and without sodium tetraborate and oxgall-bile, anti-microbial components of modified Coon's medium which did not appear to restrict growth of *L. maculans* (Experiment 1).

Medium A

¼ PDA	1000 ml
1% Erythromycin (Sigma)	10 ml
Rose Bengal (Gurr)	30 mg

Medium B

¼ PDA	1000 ml
1% Erythromycin (Sigma)	10 ml
Rose Bengal (Gurr)	30 mg
Oxgall-bile (Sigma)	0.5 g
Sodium tetraborate (BDH)	1 g

Suspensions of spores of the *L. maculans* isolates 66/97, 119/97 and 213/97 were prepared as described in section 3.2.1.4. Three 10 µl droplets of spore suspensions of each isolate were evenly spaced on plates of the Media A and B, incubated and assessed 11 days later as described above.

Experiment 3

An experiment was conducted to determine whether different light regimes had an effect on the growth of *L. maculans* on ¼ PDA supplemented with some of the anti-microbial agents tested in previous experiments. In Experiment 2, oxgall-bile or sodium tetraborate appeared to interact with Rose Bengal, reducing the intensity of pink dye. The growth of *L. maculans*

on ¼ PDA supplemented with Rose Bengal was compared to that on ¼ PDA supplemented with oxgall-bile and sodium tetraborate, components of Medium B which supported growth of *L. maculans* in Experiment 2.

Medium C	
¼ PDA	1000 ml
Rose Bengal (Gurr)	30 mg
Medium D	
¼ PDA	1000 ml
Oxgall – bile (Sigma)	0.5 g
Sodium tetraborate (BDH)	1 g

L. maculans was cultured on Media C and D under two different light regimes; 1) blacklight/fluorescent light 12 h – dark 12 h (standard light regime) and 2) fluorescent light 12 h – dark 12 h. Three single spores (replications) of isolates 66/97 or 213/97 (section 3.2.1.2) were placed on plates of both media and incubated under the respective light regimes. Cultures were assessed 12 days later as described above.

Experiment 4

An experiment was conducted to test Media A to D as selective media for enumerating *L. maculans* from soil collected from a field in which canola had been grown. Two additional media were included in this experiment. Media E and F consisted of ¼ PDA plus streptomycin sulphate with or without the fungicide, dichloran (2,6 dichloro-4-nitroaniline), which has been reported to restrict growth of *Rhizopus* and *Mucor* spp. (King *et al.* 1979; Hocking and Pitt 1980).

Medium E

¼ PDA	1000 ml
1% Streptomycin sulphate (Sigma)	10 ml

Medium F

¼ PDA	1000 ml
1% Streptomycin sulphate (Sigma)	10 ml
Dichloran (Aldrich)	2 mg

Soil was collected from a field on Eyre Peninsula, South Australia, in which canola had been grown the previous year. The extent to which the crop was affected by blackleg was unknown. The soil was diluted to 1 in 50 (2 g / 100 ml sterilised RO water) and then further diluted until 1 in 200 (soil/water) was achieved. Using three replications (three plates) of each medium (A-F), 1 ml of diluted soil suspension was placed onto the surface of each plate using a pipette, and then spread evenly with a sterile bent glass rod. Plates were incubated as in Experiment 1 and assessed for the presence of *L. maculans* 9 and 19 days later.

Experiment 5

As *L. maculans* could not be enumerated in soil using Media A to F, alternative media were evaluated. An experiment was conducted to assess the growth of *L. maculans* on two media used by King *et al.* (1979) to enumerate fungi in food. Another two media based on ¼ PDA medium were also prepared to assess further the value of dichloran and other compounds used in Media A-F.

Medium G (pH 7.2) King *et al.* (1979)

Glucose	10 g

Peptone (BDH)	5 g
Magnesium sulphate (BDH)	0.5 g
Dipotassium hydrogen phosphate (BDH)	1 g
Bacto-agar (Difco)	15 g
RO water	1000 ml
1% Erythromycin (Sigma)	10 ml
Rose Bengal (Gurr)	30 mg
Dichloran (Aldrich)	2 mg

Medium H (pH 5.6) King *et al.* (1979)

As Medium G but with potassium dihydrogen phosphate instead of dipotassium hydrogen phosphate to reduce the pH.

Medium I

¼ PDA	1000 ml
1% Erythromycin (Sigma)	10 ml
Rose Bengal (Gurr)	30 mg
Dichloran (Aldrich)	2 mg

Medium J

¼ PDA	1000 ml
1% Erythromycin (Sigma)	10 ml
Rose Bengal (Gurr)	30 mg
Oxgall-bile (Sigma)	0.5 g
Sodium tetraborate (BDH)	1 g
Dichloran (Aldrich)	2 mg

Three single spores (replications) of *L. maculans* isolates 66/97 or 213/97 were placed on a plate of each of Media G, H, I and J, incubated as in Experiment 1 and assessed 9 days later for colony diameter and presence of pycnidia.

Experiment 6

An experiment was conducted to compare three new combinations of anti-microbial agents in ¼ PDA with media on which *L. maculans* was cultured in previous experiments.

Medium K

¼ PDA	1000 ml
Dichloran (Aldrich)	2 mg
Oxgall – bile (Sigma)	0.5 g
Sodium tetraborate (BDH)	1 g
1% Erythromycin (Sigma)	10 ml

Medium L

As Medium K with 1% streptomycin sulphate instead of 1% erythromycin.

Three single spores (replications) of *L. maculans* isolates 66/97 and 213/97 were placed on a plate of each of Media K and L as well as Media B, E, G, H and I for comparison, incubated as in Experiment 1 and assessed 9 days later for the presence of mycelia and pycnidia.

Experiment 7

In previous experiments, mycelial growth of *L. maculans* and pycnidium formation occurred on media K and L, so this experiment was designed to assess the potential of both media to eliminate other soil microbes. Soil was collected from near a creek (i.e. never sown to crops) on the Charlick Research Station and inoculated with *L. maculans* as follows. A suspension

of 10^6 pycnidiospores /ml of *L. maculans* isolate 66/97 was prepared as in section 3.2.1.4, and 5 ml was added to 20 g of soil then mixed thoroughly. A sub-sample of 2 g was diluted in 100 ml sterile RO water (1 in 50 dilution). Using a magnetic stirrer to keep the spores in suspension, 50 ml was removed using a 25 ml burette with a bulb on top (Crown Scientific) and added to 50 ml of sterile RO water to make a 1 in 100 dilution. This was repeated a number of times until a dilution of 1 in 1,600 was achieved. Using a pipette, 0.5 ml of each dilution was placed onto two plates (replications) of each media, K and L. The suspensions were spread evenly over the plates as in Experiment 4. The plates were incubated as in Experiment 1 and assessed 8 days later for the presence of *L. maculans*. Subcultures were made from putative colonies of *L. maculans* onto fresh plates for identification based on Punithalingam and Holliday (1972).

Experiment 8

As *L. maculans* in artificially inoculated soil could be enumerated on Media K and L in Experiment 7, this experiment was designed to test the suitability of both media for use in enumerating *L. maculans* in naturally infested soil. Soil from Eyre Peninsula, as in Experiment 4, collected 1 year after canola was grown, was diluted as in Experiment 7 and then 0.5 ml of each dilution was spread onto four plates of each medium, as in Experiment 4. Plates were incubated as in Experiment 1 and assessed 9 days later for the presence of *L. maculans* as described in Experiment 7.

Experiment 9

As Medium K was somewhat less conducive to the growth of soil microorganisms than was Medium L in Experiment 8, the former was assessed for suitability in enumerating *L. maculans* in stubble. Canola stubble, comprising various cultivars, bearing pycnidia of *L. maculans* was obtained in 1999 from a canola blackleg trial managed by Mr Trent Potter,

SARDI, Struan Agricultural Centre, and milled in a laboratory mill (Thomas-Wiley) with a 2 mm sieve. A 2 g sub-sample of milled stubble collected through the sieve was added to 100 ml sterile RO water and diluted six times, as in Experiment 7, then 0.5 ml of each dilution was spread over four plates of Medium K (replications) as in Experiment 4. Plates were incubated as in Experiment 1 and assessed 14 days later for the presence of *L. maculans* as described in Experiment 7.

Experiment 10

This experiment was designed to compare the growth of *L. maculans*, on Medium K, from infested stubble which had been milled as described in Experiment 9 and stubble which had been milled and subsequently ground to a rough powder using an automated mortar and pestle. The two treatments of stubble will be referred to as "milled" and "ground", respectively. "Milled" and "ground" stubbles were diluted as in Experiment 7 and plated on medium K, using four replications per dilution. Plates were incubated as in Experiment 1 and assessed 12 days later for the presence of *L. maculans* as described in Experiment 7.

Experiment 11

A plant bioassay was designed to compare the infectivity of "milled" and "ground" stubble, in order to determine the most appropriate means of stubble preparation. Eight punnets (900 ml each) were placed in each of three trays and filled with potting soil (section 3.1.2.1). Three seeds of cv. Hyola 42 were sown in each punnet and later thinned to two seedlings per punnet. Once the plants reached GS 2.3, all plants were misted with a spray bottle containing RO water and Tween surfactant. One tray was evenly dusted with 4 g of "ground" stubble which was shaken over the plants, the second with 4 g of "milled" stubble and the third left untreated as a control. The three trays were placed into separate clear polythene bags for 4 days on a bench in the glasshouse (see Figure 4.1B), and then bags were removed and plants maintained

as in section 3.1.2.3. Plants were assessed 19 days after inoculation by counting the number of leaf lesions on all plants in a tray.

7.2.2 Validation of DNA-based assay for diagnostic purposes

In this section, fractions of canola stubble in oaten hay are referred to as; 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512, and dilutions of stubble suspensions in RO water are referred to as; 1 in 50, 1 in 100, 1 in 200, 1 in 400, 1 in 800, 1 in 1,600, 1 in 3,200, 1 in 6,400, 1 in 12,800, 1 in 25,600, 1 in 51,200, 1 in 102,400 and 1 in 204,800.

7.2.2.1 Stubble preparation

Fractions of infected canola stubble in oaten hay were prepared for an experiment in which the aim was to examine the correlation between the semi-selective Medium K, the bioassay and DNA analysis.

"Milled" canola stubble (80g) was prepared from stubble collected at the blackleg disease trial at Struan (as described in Experiment 9, section 7.2.1). Half of the "milled" canola stubble (40 g) was ground to a rough powder using an automated mortar and pestle. Oaten hay was collected from a field on the Charlick Research Station in November 2000 and 360 g were milled. Half (180 g) of the "milled" oaten hay was ground to a rough powder using an automated mortar and pestle. Both "milled" and "ground" oaten hay was then autoclaved for 15 min at 121°C at above 120 kPa.

Twenty grams of "milled" canola stubble were mixed with 20 g of "milled" oaten hay and shaken by hand for several minutes in a sealed plastic container, to produce a 1/2 fraction (canola/total). Twenty grams of this mixture were then combined with another 20 g of "milled" oaten hay and shaken for several minutes to produce a 1/4 fraction. This procedure

was repeated nine times, producing a series of ten fractions; 100% canola, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256 and 1/512 (canola/total). This procedure was repeated for the "ground" canola stubble and oaten hay.

7.2.2.2 Most probable number of colony forming units in stubble

The MPN of *L. maculans* CFU in each canola stubble fraction was determined. Plates containing Medium K were prepared as in Experiment 6 (section 7.2.1). Medium K was chosen out of all the media tested (section 7.2.1) as it was used in the successful enumeration of *L. maculans* from infested canola stubble. Each canola stubble fraction (section 7.2.2.1) and 100% oaten hay were diluted in the following way. Two grams of "ground" sample were added to 100 ml sterile RO water in a beaker containing a magnetic stirrer to keep the suspension homogeneous. Using a 25 ml burette with bulb on top (Crown Scientific), 50 ml of suspension was added to 50 ml sterile RO water in another beaker, also containing a magnetic stirrer. This process was repeated until 10 dilutions were achieved for each of the following stubble fractions; 1/8, 1/16, 1/64, 1/128, 1/256, 1/512, 100% oaten hay and 13 dilutions were achieved for each of the following stubble fractions; 100% canola, 1/2, 1/4. Using a pipette, a 0.5 ml aliquot of each suspension was placed on four plates (replications) containing Medium K and spread evenly as in Experiment 4 (section 7.2.1). Thirteen days after inoculation, plates were assessed for the presence of *L. maculans* as described in Experiment 7 in section 7.2.1.

For each stubble fraction, the total number of plates which yielded *L. maculans* was recorded. Using the table in Appendix 15, the number (m) of *L. maculans* spores or propagules in each sample was ascertained. Then to account for the highest dilution (d) at which colonies appeared and volume of aliquot (v) in determining the MPN of colony forming units of *L. maculans* per gram of stubble, the following equation was used:

Equation 7.1: $MPN = \frac{m\,d}{v}$ (Somasegaran and Hoben 1985)

7.2.2.3 Bioassay

The potential infectivity of stubble samples was determined in an experiment in the glasshouse. Punnets (900 ml, nine per tray) were filled with potting soil (section 3.1.2.1). Three seeds of cv. Hyola 42 were sown into each punnet, and later thinned to one plant per punnet. Once plants reached GS 2.3, they were misted with RO water and Tween surfactant (0.05%) to enable inoculum to adhere to the leaf surface. Each tray was inoculated with one stubble fraction (100% canola, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256, 1/512 (canola/total) and 100% oaten hay) and a control was left uninoculated. The "milled" stubble treatments (4 g) were dusted evenly over the plants by shaking from a McCartney bottle. Each tray containing punnets with plants was incubated as described in Experiment 11 in section 7.2.1. The experiment was arranged as a randomised complete block design with four replications. The number of leaf lesions was counted on each plant 15 days after inoculation.

7.2.2.4 DNA extraction

To prepare samples for DNA analysis, DNA was extracted in the following way. Each "ground" stubble fraction was ground to a fine powder in liquid nitrogen, using a mortar and pestle. DNA was extracted from 0.1 g of each sample using a MoBio Ultra Clean™ soil DNA kit, using the standard protocol as detailed in the instruction manual (MoBio catalogue number: 12800-100-S, Lot #SD8G27, MoBio Laboratories, Inc., California, USA). The amount of *L. maculans* DNA in each stubble fraction was then estimated using a DNA-based assay involving primers specific for *L. maculans* (RDTS, SARDI).

7.2.2.5 Correlation of the data

The relationship between the results from the DNA-based assay and those from the dilution series on Medium K and bioassay was assessed by linear regression and the coefficient of determination (R^2) was calculated as described by Herdina and Roget (2000).

7.2.3 Determination of sampling requirements

Field sampling was conducted to determine the requirements necessary for accurate soil sampling and was based on the method of Herdina and Roget (2000). On April 6 2000, soil was collected from the 'Ruradene' property, which is located 2 km south of Tarlee in the mid-north region of South Australia and managed by John and Ange Rohde. Two fields were chosen based on canola rotation; R9 – canola grown in the previous year and Co1 - canola grown 2 years earlier. Both fields contained red-brown earth soil (see Appendix 16) and the extent of blackleg infection on the previous canola crops was unknown.

A grid (90 m x 90 m or 0.81 ha) was marked out in both fields with wooden stakes as shown in Appendix 17, with seven rows (15 m apart) and seven samples (15 m apart) per row per site. A total of 49 sub-samples of approximately 300 g was taken from the gridline intersections. At each sampling point, a quadrat (one-tenth of a square metre) was placed on the ground and nine soil cores (10 cm x 2 cm, volume 31.43 cm^3) were taken from within the quadrat (Figure 7.1A&B). Soil cores from each sampling point were combined into one bag, labelled and returned to the laboratory where they were stored in a cold room at 4°C overnight. The following day, the soil was spread out in trays and dried in an oven (40°C) for 1 week, mixing every second day with a clean trowel. The amount of *L. maculans* inoculum in each sub-sample was then estimated using the DNA-based assay (RDTS, SARDI).

The upper and lower confidence limits at 95% (CL^+ or CL^-), means, standard deviation, minimum, median and maximum values for amount of *L. maculans* DNA were calculated for all 49 individual sub-samples and then for selections of 37, 28, 16 and 8 sub-samples, from the grid pattern. The number of sub-samples from a field (sampling intensity) which was necessary to obtain a representative sample was determined.

Figure 7.1: A. Sampling soil in a field with a soil corer within a quadrat. B. Nine soil cores were removed from within the quadrat at each sampling point.

7.3 RESULTS

7.3.1 Development of selective medium for *L. maculans*

Experiment 1

Growth of *L. maculans* isolates 66/97 and 213/97 on plates of modified Coon's medium was assessed 8 days after inoculation. Growth was very slow and no pycnidia formed. *L. maculans* grew normally on plates of modified Coon's medium without antibiotics and pycnidia had begun to form after 7 days. On further assessment at 14 days, growth had not progressed. The results from assessment of cultures on modified Coon's medium with each antibiotic individually are shown in Table 7.1. Mycelial growth was restricted on the medium containing PCNB and appeared normal on media with the other three anti-microbial agents, but pycnidia did not form in the presence of any of these agents.

Table 7.1: Mean diameter (mm) of colonies 13 days after placing storage plugs of three *L. maculans* isolates on plates of modified Coon's medium (see section 7.2.1) with four different anti-microbial agents individually (given per 1000 ml of medium). Each treatment was replicated three times.

Isolate	Oxgall 0.5 g	PCNB 1 g	Erythromycin 10 ml	Sodium tetraborate 1 g
213/97	20	7	27	32
119/97	30	6	50	47
66/97	25	4	38	32

Experiment 2

The results of growth of *L. maculans* on Media A and B are shown in Table 7.2. Mycelium grew on Medium B but not on Medium A. The pink pigment of Rose Bengal appeared to be less intense in Medium B than in Medium A. All three isolates produced pycnidia on Medium B, with 66/97 producing the most.

Table 7.2: Diameter (mm) of colonies 11 days after placing three 10 µl droplets of three *L. maculans* isolates on two different media with three replications (R) per treatment.

Isolate	Medium A			Medium B		
	R1	R2	R3	R1	R2	R3
66/97	0	0	0	28	36	40
119/97	0	0	0	40	40	40
213/97	0	0	0	35	35	40

Anti-microbial agents (per 1000 ml of ¼ PDA): Medium A – Erythromycin (10 ml) + Rose Bengal (30 mg), Medium B – Erythromycin (10 ml) + Rose Bengal (30 mg) + Oxgall – bile (0.5 g) + Sodium tetraborate (1 g)

Experiment 3

No hyphal growth was observed on Medium C. On Medium D, mycelium was produced in one of three replicates of the A-group isolate 66/97, on plates exposed to both light regimes (Table 7.3). Mycelium was produced in all three replicates of the B-group isolate 213/97 which were exposed to both light regimes, but pycnidia formed only in cultures exposed to light regime 1.

Table 7.3: Diameter (mm) of colonies 12 days after single spores of two *L. maculans* isolates were placed on plates of two different media with three replications (R) per treatment.

	Light regime 1						Light regime 2					
Isolate	66/97			213/97			66/97			213/97		
	R1	R2	R3	R1	R2	R3	R1	R2	R 3	R1	R2	R3
Medium C	0	0	0	0	0	0	0	0	0	0	0	0
Medium D	32[2]	0	0	40[1]	35[1]	32[1]	25[2]	0	0	55[2]	50[2]	50[2]

[1] some pycnidia formed, [2] no pycnidia formed

Light regime 1 - blacklight/fluorescent 12 h / dark 12 h, Light regime 2 - fluorescent 12 h / dark 12 h
Anti-microbial agents (per 1000 ml ¼ PDA): Medium C – Rose Bengal (30 g), Medium D – Oxgall-bile (0.5 g) + Sodium tetraborate (1 g)

Experiment 4

Nine days after inoculation, no colonies of *L. maculans* could be detected on plates of media A, B, C, D, E and F, but there were some colonies of other soil microorganisms present. Nineteen days after inoculation, no *L. maculans* was detected on any of the plates and media B, C, D and E were overgrown with other soil microorganisms, whereas plates of media A and F had less microbial growth.

Experiment 5

L. maculans isolates 66/97 and 213/97 did not grow on media H and J, and only single restricted colonies of isolate 213/97 grew on medium G (15 mm diameter) and medium I (5 mm diameter), with no pycnidia produced. Media H-J all contained the anti-microbial agents; erythromycin (10 ml per 1000 ml of ¼ PDA), Rose Bengal (30 mg) and Dichloran (2 mg) and Medium J also contained oxgall-bile (0.5 g) and sodium tetraborate (1 g).

Experiment 6

No colonies grew on media B, E, G and H (Table 7.4). On Medium I, all but one replication of isolate 66/97 formed mycelium without any pycnidium formation. On Medium K, mycelium and pycnidia formed for all three replicates of isolate 66/97 but for only one replicate of isolate 213/97. On medium H, mycelium grew for one replicate each of isolates 66/97 and 213/97, but only 213/97 formed pycnidia.

Table 7.4: Presence or absence of mycelium and pycnidia of two *L. maculans* isolates (66/97 A-group and 213/97 B-group) on a range of media with three replications (R) per treatment.

Medium	Isolate 66/97			Isolate 213/97		
	R1	R2	R3	R1	R2	R3
B	-	-	-	-	-	-
E	-	-	-	-	-	-
G	-	-	-	-	-	-
H	-	-	-	-	-	-
I	h	h	-	h	h	h
K	h,p	h,p	h,p	h,p	-	-
L	h	-	-	h,p	-	-

hyphal growth (h), pycnidia (p), no growth (-)

Summary of growth of L. maculans on Media A-L

Table 7.5 summarises the ability of *L. maculans* to form mycelium and pycnidia on the putative selective media assessed. Hence, media which did not support growth of the fungus were eliminated. However, *L. maculans* did not grow from naturally infested soil dilutions on Media B and D (Experiment 4), so only Media K and L were considered further.

Table 7.5: Summary of the presence (+) or absence (-) of mycelial growth and pycnidia of *L. maculans* on the various "selective media" assessed, when inoculated with pure cultures of the fungus.

Media	Ingredients	Mycelium	Pycnidia
Modified Coon's medium	Coon's medium, Eryth, Ox, PCNB, Na_2BO_4	+	-
Medium A	¼ PDA, Eryth, RB	-	-
Medium B	¼ PDA, Eryth, RB, Ox, Na_2BO_4	+	+
Medium C	¼ PDA, RB	-	-
Medium D	¼ PDA, Ox, Na_2BO_4	+	+
Medium E	¼ PDA, Strep	-	-
Medium F	¼ PDA, Strep, Dc	-	-
Medium G	Gluc, Pep, $MgSO_4$, BA, Eryth, RB, Dc, K_2HPO_4	+	-
Medium H	Gluc, Pep, $MgSO_4$, BA, Eryth, RB, Dc, KH_2PO_4	-	-
Medium I	¼ PDA, Eryth, RB, Dc	+	-
Medium J	¼ PDA, Eryth, RB, Dc, Ox, Na_2BO_4	-	-
Medium K	¼ PDA, Eryth, Dc, Ox, Na_2BO_4	+	+
Medium L	¼ PDA, Strep, Dc, Ox, Na_2BO_4	+	+

¼ PDA = quarter strength potato dextrose agar, Eryth = erythromycin, Strep = streptomycin sulphate, Ox = oxgall-bile, PCNB = pentachloronitrobenzene, Na_2BO_4 = Sodium tetraborate, RB = Rose Bengal, Dc = Dichloran, Gluc = glucose, Pep = peptone, $MgSO_4$= Magnesium sulphate, K_2HPO_4= Dipotassium hydrogen phosphate, KH_2PO_4 = Potassium dihydrogen phosphate, BA = bacto agar

Experiment 7

Colonies of *L. maculans* were identified by macroscopic and microscopic morphology (Punithalingham and Holliday 1972) on both Medium K and L in some dilutions of the inoculated soil (Table 7.6). It was also observed that there was far less growth of other soil bacteria and fungi on Medium K than on Medium L. There appeared to be a wide range of soil microorganisms including *Rhizopus, Penicillium* and *Trichoderma* spp.

Table 7.6: Presence (+) and absence (-) of colonies of *L. maculans* on two culture media. Soil was artificially inoculated with pycnidiospores of *L. maculans* isolate 66/97, serial dilutions prepared in sterile RO water and 0.5 ml of each suspension was spread over the surface of plates with two replications (R) per treatment.

	Medium K		Medium L	
Dilution	R1	R2	R1	R2
1 in 50	-	-	+	+
1 in 100	-	-	-	+
1 in 200	-	+	+	-
1 in 400	+	+	+	-
1 in 800	-	-	-	+
1 in 1600	-	+	-	-

Anti-microbial agents (per 1000 ml of ¼ PDA): Dichloran (2 mg) + oxgall-bile (0.5 g) + sodium tetraborate (1 g) + erythromycin (10 ml, Medium K only) or streptomycin sulphate (10 ml, Medium L only)

Experiment 8

When inoculated with dilutions of soil naturally infested with *L. maculans*, all plates of Media K and L were heavily overgrown with a wide range of soil microorganisms (as described in Experiment 7). Medium K appeared to have less growth than Medium L. Only two plates (dilutions 1 in 400 and 1 in 800) of Medium K were identified to contain *L. maculans*.

Experiment 9

Table 7.7 reveals that *L. maculans* colonies were identified on plates of medium K, which had been inoculated with a series of dilutions of "milled" infested canola stubble. However, plates were overgrown with other fungi and bacteria which naturally infest canola stubble, making identification difficult.

Table 7.7: Presence (+) or absence (-) of colonies of *L. maculans* on medium K inoculated with a series of dilutions of "ground" infected canola stubble with four replications (R) per dilution. Ingredients in Medium K : ¼ PDA (1000 ml) + Dichloran (2 mg) + oxgall-bile (0.5 g) + sodium tetraborate (1 g) + erythromycin (10 ml).

Dilution	R1	R2	R3	R4
1 in 50	+	+	+	+
1 in 100	+	+	+	+
1 in 200	+	+	-	+
1 in 400	+	+	+	+
1 in 800	-	+	-	+
1 in 1600	+	-	+	-

Experiment 10

Table 7.8 shows the results of dilution plating with both "milled" and "ground" stubble. It was possible to identify colonies of *L. maculans* on every plate that had been inoculated with "ground" stubble. Colonies were identified only on plates which received the last two dilutions (1 in 800 and 1 in 1600) of the "milled" stubble, as all other plates were completely overgrown by a wide range of other microorganisms.

Table 7.8: Presence (+) or absence (-) of colonies of *L. maculans* on medium K inoculated with a series of dilutions of "milled" and "ground" infected canola stubble with four replications (R) per dilution. Ingredients in Medium K : ¼ PDA (1000 ml) + Dichloran (2 mg) + oxgall-bile (0.5 g) + sodium tetraborate (1 g) + erythromycin (10 ml).

	"Ground" stubble				"Milled" stubble			
Dilution	R1	R2	R3	R4	R1	R2	R3	R4
1 in 50	+	+	+	+	-	-	-	-
1 in 100	+	+	+	+	-	-	-	-
1 in 200	+	+	+	+	-	-	-	-
1 in 400	+	+	+	+	-	-	-	-
1 in 800	+	+	+	+	+	+	+	+
1 in 1600	+	+	+	+	+	+	+	+

Experiment 11

In the preliminary bioassay, there were 43 lesions typical of blackleg counted on the leaves of plants that were dusted with "milled" stubble, three leaf lesions formed on the plants that were dusted with "ground" stubble and no leaf lesions formed on the control plants.

7.3.2 Validation of DNA-based assay for diagnostic purposes

Table 7.9 shows determination of MPN of CFU of *L. maculans* in the various fractions of stubble based on presence or absence of the fungus on four replicate plates inoculated with each dilution. Using Equation 7.1, the MPN of CFU of *L. maculans* in each canola stubble fraction was calculated (Tables 7.9 and 7.10). The MPN of CFU decreased from 43,200 when inoculated with 100% canola to zero when inoculated with a dilution higher than 1/128 (canola /total).

The plants of Hyola 42 which had been inoculated with the various fractions of stubble were assessed 15 days after inoculation by counting the total number of leaf lesions on the nine plants in each tray. Table 7.10 shows the mean leaf lesion counts on plants treated with each canola stubble fraction, the 100% oaten hay sample and the uninoculated control. The mean number of leaf lesions per nine plants decreased from 123 when inoculated with 100% milled canola stubble to less than one when inoculated with 100% oats or left uninoculated as a control.

Table 7.9: Presence (+) or absence (-) of *L. maculans* on plates of Medium K 13 days after inoculation with a series of dilutions of "ground" canola stubble fractions[a]. The most probable number (MPN) of colony forming units (CFU) of *L. maculans* in each stubble fraction was calculated using Equation 7.1. Each dilution is replicated (R) on four separate plates.

Dilution	R1	R2	R3	R4	Count
100% canola					
1 in 400	+	+	+	+	4
1 in 800	+	+	+	+	4
1 in 1,600	+	+	+	+	4
1 in 3,200	+	+	+	+	4
1 in 6,400	+	+	+	+	4
1 in 12,800	+	+	+	+	4
1 in 25,600	+	-	+	+	3
1 in 51,200	-	-	-	-	0
1 in 102,400	-	-	-	-	0
1 in 204,800	-	+	-	-	1
				total	28
MPN	43,200 CFU / g stubble				
1/2 canola					
1 in 400	+	+	+	+	4
1 in 800	+	+	+	+	4
1 in 1,600	+	+	+	+	4
1 in 3,200	+	+	+	+	4
1 in 6,400	-	-	+	-	1
1 in 12,800	-	-	+	-	1
1 in 25,600	-	-	-	+	1
1 in 51,200	+	-	-	-	1
1 in 102,400	-	-	-	-	0
1 in 204,800	-	-	-	+	1
				total	21
MPN	12,000 CFU / g stubble				
1/4 canola					
1 in 400	+	+	+	+	4
1 in 800	+	+	+	+	4
1 in 1,600	+	+	+	+	4
1 in 3,200	+	+	+	-	4
1 in 6,400	+	-	+	+	3
1 in 12,800	+	-	-	-	1
1 in 25,600	-	-	-	-	0
1 in 51,200	-	-	-	-	0
1 in 102,400	-	-	-	-	0
1 in 204,800	-	-	-	-	0
				total	19
MPN	8,800 CFU / g stubble				

Dilution	R1	R2	R3	R4	Count
1/8 canola					
1 in 50	+	+	+	+	4
1 in 100	+	+	+	+	4
1 in 200	+	+	+	+	4
1 in 400	+	+	+	+	4
1 in 800	+	+	+	+	4
1 in 1,600	+	+	+	+	4
1 in 3,200	+	+	+	-	3
1 in 6,400	-	-	-	-	0
1 in 12,800	-	-	+	-	1
1 in 25,600	-	-	-	-	0
				total	28
MPN	5,400 CFU / g stubble				
1/16 canola					
1 in 50	+	+	+	+	4
1 in 100	+	+	+	+	4
1 in 200	+	+	+	-	3
1 in 400	-	+	+	-	2
1 in 800	+	-	+	-	2
1 in 1,600	-	+	-	+	2
1 in 3,200	-	-	-	-	0
1 in 6,400	-	-	-	-	0
1 in 12,800	-	-	-	-	0
1 in 25,600	-	+	-	-	1
				total	18
MPN	890 CFU / g stubble				
1/32 canola					
1 in 50	+	+	+	+	4
1 in 100	+	+	+	+	4
1 in 200	+	-	+	+	3
1 in 400	+	+	+	-	3
1 in 800	-	-	+	+	2
1 in 1,600	-	+	+	-	2
1 in 3,200	-	+	+	-	2
1 in 6,400	+	-	-	-	1
1 in 12,800	-	-	-	-	0
1 in 25,600	-	-	-	-	0
				total	21
MPN	1,500 CFU / g stubble				

(continued)

Table 7.9: continued

Dilution	R1	R2	R3	R4	Count
1/64 canola					
1 in 50	+	-	-	+	2
1 in 100	-	-	+	-	1
1 in 200	-	-	-	+	1
1 in 400	-	-	-	-	0
1 in 800	-	-	-	-	0
1 in 1,600	-	-	-	-	0
1 in 3,200	-	-	-	-	0
1 in 6,400	-	-	-	-	0
1 in 12,800	-	-	-	-	0
1 in 25,600	-	-	-	-	0
				total	**4**
MPN	**120 CFU /g stubble**				
1/128 canola					
1 in 50	-	+	-	-	1
1 in 100	-	+	-	-	1
1 in 200	-	-	-	-	0
1 in 400	-	-	-	-	0
1 in 800	-	-	-	-	0
1 in 1,600	-	-	-	-	0
1 in 3,200	-	-	-	-	0
1 in 6,400	-	-	-	-	0
1 in 12,800	-	-	-	-	0
1 in 25,600	-	-	-	-	0
				total	**2**
MPN	**54 CFU / g stubble**				
1/256 canola					
1 in 50	-	-	-	-	0
1 in 100	-	-	-	-	0
1 in 200	-	-	-	-	0
1 in 400	-	-	-	-	0
1 in 800	-	-	-	-	0
1 in 1,600	-	-	-	-	0
1 in 3,200	-	-	-	-	0
1 in 6,400	-	-	-	-	0
1 in 12,800	-	-	-	-	0
1 in 25,600	-	-	-	-	0
				total	**0**
MPN	**0 CFU / g stubble**				

Dilution	R1	R2	R3	R4	Count
1/512 canola					
1 in 50	-	-	-	-	0
1 in 100	-	-	-	-	0
1 in 200	-	-	-	-	0
1 in 400	-	-	-	-	0
1 in 800	-	-	-	-	0
1 in 1,600	-	-	-	-	0
1 in 3,200	-	-	-	-	0
1 in 6,400	-	-	-	-	0
1 in 12,800	-	-	-	-	0
1 in 25,600	-	-	-	-	0
				total	**0**
MPN	**0 CFU / g stubble**				
100% oats					
1 in 50	-	-	-	-	0
1 in 100	-	-	-	-	0
1 in 200	-	-	-	-	0
1 in 400	-	-	-	-	0
1 in 800	-	-	-	-	0
1 in 1,600	-	-	-	-	0
1 in 3,200	-	-	-	-	0
1 in 6,400	-	-	-	-	0
1 in 12,800	-	-	-	-	0
1 in 25,600	-	-	-	-	0
				total	**0**
MPN	**0 CFU / g stubble**				

a: 100% canola = canola only; 1/2 canola = canola:oats (1:1); 1/4 canola = canola:oats (1:3); 1/8 canola = canola:oats (1:7); 1/16 canola = canola:oats (1:15); 1/32 canola = canola:oats (1:31); 1/64 canola = canola:oats (1:63); 1/128 canola = canola:oats (1:127); 1/256 canola = canola:oats (1:255); 1/512 canola = canola:oats (1:511) and 100% oats = oats only.

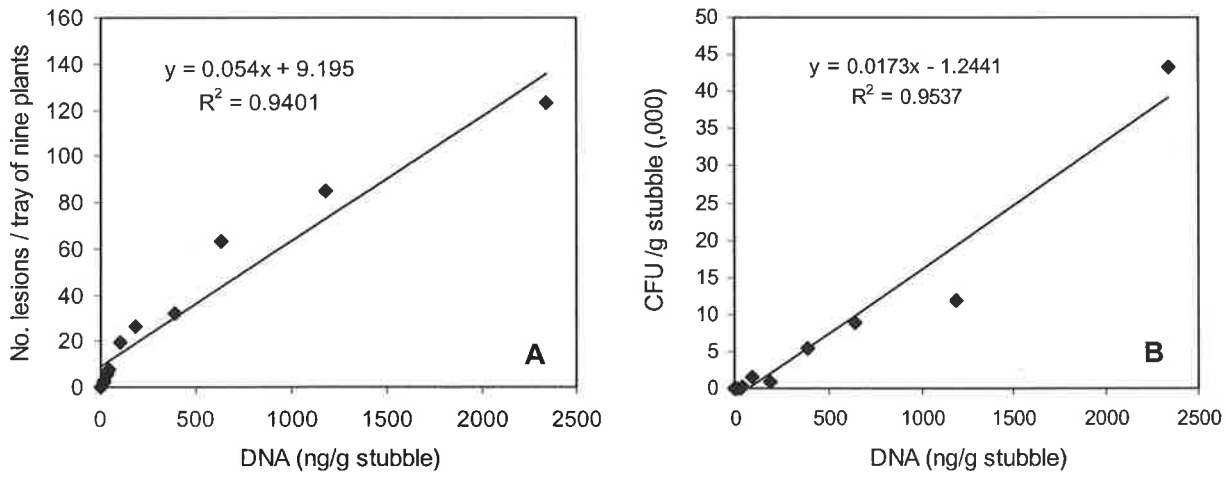
The quantities of *L. maculans* DNA estimated by means of the DNA-based assay (RDTS, SARDI) are shown in Table 7.10. As the quantity of *L. maculans* DNA increased, so too did the number of blackleg leaf lesions per nine plants and the MPN of CFU of the fungus. The quantity of DNA was correlated closely with mean number of leaf lesions per nine plants (R^2 = 0.94, Figure 7.2A) and MPN of colony forming units (R^2 = 0.95, Figure 7.2B).

Table 7.10: Series of canola stubble fractions* with MPN of CFU of *L. maculans* diluted and plated on a semi-selective medium, mean number of leaf lesions on nine plants per tray in a bioassay and quantity of *L. maculans* DNA as determined by a DNA assay (RDTS, SARDI).

Stubble fraction* (canola/total)	MPN# (x 100 CFU)	Mean no. of leaf lesions / tray^	*L. maculans* DNA (ng / g stubble)
100% canola	432.0	123.3^{a}	2335.6
1/2	120.0	84.8^{b}	1182.7
1/4	88.0	63.3^{c}	639.9
1/8	54.0	32.3^{d}	393.0
1/16	8.9	26.3^{de}	189.3
1/32	15.0	19.8^{def}	97.8
1/64	1.2	8.0^{efg}	45.9
1/128	0.5	5.3^{fg}	34.0
1/256	0.0	3.0^{fg}	12.8
1/512	0.0	2.3^{fg}	13.6
100% oats	0.0	0.3^{g}	0.5
control		0.8^{g}	

* canola stubble "milled" and mixed with "milled" autoclaved oaten hay to produce fractions
#MPN (the most probable number) of CFU (colony forming units) as in Table 7.9
^ Values with the same superscript are not significantly different ($LSD_{0.05}$)

Figure 7.2: Correlation of quantity of *L. maculans* DNA (estimated by the RDTS, SARDI) with A. mean number of leaf lesions per tray in a canola plant bioassay and B. most probable number of *L. maculans* colony forming units on dilution plates of Medium K.



7.3.3 Determination of sampling requirements

Results for estimated quantities of *L. maculans* DNA, provided by the RDTS, SARDI, in the sub-samples of soil from field R9 are shown in Table 7.11. It reveals the variation between estimated DNA levels in the sub-samples of soil taken from gridline intersections. Values ranged from 4 to 3,822 pg/g soil with no obvious pattern of distribution. *L. maculans* DNA could not be detected in any of the sub-samples from field Co1 and it was assumed that if the fungus was present it was at low levels.

The statistical results for the sampling intensities (number of sub-samples taken from a field) are shown in Table 7.12. There was a considerable increase in the standard deviation as the number of sub-samples decreased. Figure 7.3 shows the expected increase in error in the estimation of amount of DNA of *L. maculans* as the number of sub-samples contributing to the mean value decreased. The increase in error was most notable when sub-sample number was below 30. Therefore, more than 30 sub-samples would need to be taken from a field to be confident of obtaining a meaningful result.

Table 7.11: The estimated quantity of *L. maculans* DNA (pg/g soil) for each of the 49 sub-samples, taken in April 2000, from the intersections of a grid pattern (90 m x 90 m, Appendix 17) in a field (R9) near Tarlee, South Australia, into which canola had been sown in 1999.

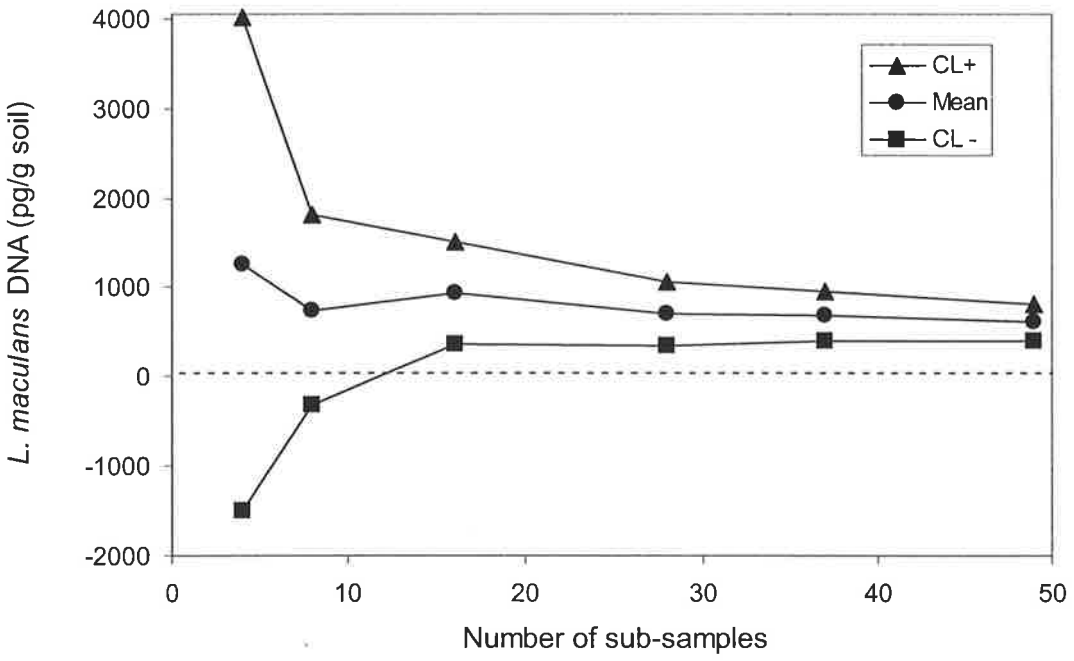
	1	2	3	4	5	6	7
A	63	49	434	23	3822	583	710
B	541	592	30	334	385	1511	391
C	2400	1855	385	121	2258	655	450
D	271	128	218	381	725	470	88
E	48	68	292	396	548	307	24
F	93	1008	758	543	1159	386	4
G	1045	123	1831	391	460	214	199

Table 7.12: Statistical results for different sampling intensities (number of sub-samples taken from a field) at intersections of a grid pattern (90 m x 90 m, Appendix 17) in a field which had canola sown in the previous year, near Tarlee, South Australia. Quantity of *L. maculans* DNA at each sampling point was estimated by the RDTS, SARDI. CL refers to the confidence limit at 95%.

	No. of sub-samples					
	49	37	28	16	8	4
Lower 95% CL	396.2	407.1	352.6	358.3	-318.9	-1496.5
Mean[a]	607.5	678.5	705.5	935.5	742.5	1257.2
Upper 95% CL	818.8	949.9	1058.4	1512.7	1804.0	4011.0
Standard Deviation	735.7	814.0	910.1	1083.2	1269.7	1730.6
Minimum	3.6	23.0	23.0	23.7	23.7	62.5
Median	390.7	395.7	393.2	454.7	363.0	572.0
Maximum	3822.4	3822.4	3822.4	3822.4	3822.4	3822.4

a: mean estimated quantity of *L. maculans* DNA (pg/g soil)

Figure 7.3: The influence of sampling intensity (number of sub-samples taken from a field) on error in assessments of *L. maculans* DNA in a field (R9) near Tarlee, South Australia. A total of 49 soil samples of 300 g was collected from a grid (90m x 90m) and submitted to the RDTS, SARDI for DNA assay. CL+ refers to the upper confidence limit at 95%. CL- refers to the lower confidence limit at 95%. Mean refers to the mean estimated quantity of *L. maculans* DNA (pg/g soil).



7.4 DISCUSSION

The development of a medium to select for *L. maculans* and inhibit as many other fungi and bacteria as possible, was based on media used previously to isolate other pathogens from soil and food. Coon's agar medium was initially chosen because of its suitability as a selective medium for *M. pinodes* and *P. medicaginis* var. *pinodella* (Davidson *et al.* 1999), but *L. maculans* grew very slowly on this medium and pycnidia did not develop. As pycnidia are required for identification of the fungus amongst other fungal colonies, Coon's agar medium was not considered further.

The commonly used medium, ¼ PDA, was also assessed for suitability as the base for a selective medium since it has been successfully used for culturing *L. maculans*, allowing rapid hyphal growth and abundant pycnidial formation (section 3.2.1, Sosnowski 1999; Sosnowski *et al.* 2001b). The addition of Rose Bengal for its selective anti-fungal properties against *Rhizopus* and *Mucor* spp. (King *et al.* 1979; Dhingra and Sinclair 1995) inhibited the growth of *L. maculans* in Medium A and C, but the fungus grew and produced some pycnidia in medium B, which had less pink pigment than the other two media. There may have been an interaction between either the oxgall-bile or sodium tetraborate and the Rose Bengal, which reduced the anti-fungal effect of Rose Bengal in medium B.

Thirteen different combinations of anti-microbial compounds in ¼ PDA were assessed by placing single-spores of *L. maculans* on plates of media and assessing hyphal growth and pycnidial formation. Four of the media (B, D, K and L) supported hyphal growth and formation of pycnidia. When soil taken from a field in which canola had been grown in the previous year was diluted in water and spread over the four media, excessive overgrowth by other soil microorganisms made it very difficult to detect *L. maculans* colonies. The extent of blackleg infection in the previous canola crop was unknown. However, there was less growth

of other soil organisms on Medium K than on Media B, D and L (Experiments 4 and 8) and so Medium K was selected for further development as a selective medium. The anti-microbial components of Medium K which appeared to be important in its ability to inhibit soil organisms more effectively than the other media are erythromycin and Dichloran, since all four media contained oxgall-bile and sodium tetraborate, neither Media B and D contained Dichloran and Medium L contained streptomycin sulphate instead of erythromycin. It seems that erythromycin may inhibit a broader spectrum of soil bacterial species than streptomycin sulphate. Of 31 anti-fungal compounds tested by King *et al.* (1979), Dichloran was reported to play a major role in the restriction of *Rhizopus* and *Mucor* spp. while allowing growth of mycotoxigenic moulds and other fungal species of significance in food spoilage.

The RDTS, SARDI routinely grinds soil and stubble samples to a rough powder, using an automated mortar and pestle, for use in DNA analysis. When ground canola stubble was diluted in sterile water and spread over medium K, colonies of *L. maculans* could be identified, and were not overgrown by other fungi. However, when ground canola stubble was spread over canola plants in the preliminary bioassay, very few leaf lesions developed after incubation for 19 days. Canola stubble that was milled through a 2-mm sieve, then spread over canola plants produced a sufficient number of leaf lesions to enable comparison between different samples of stubble. This suggested that fungal spores and hyphae may be damaged in the grinding process, reducing their ability to infect canola leaves. However, on the surface of the nutrient-rich culture medium, the damaged fungus could grow from ground stubble. Hence, for validation of the DNA assay, milled canola stubble was used for the bioassay and stubble which was milled and then ground was used for the selective medium and DNA assay.

There was a strong statistical correlation between the quantity of *L. maculans* DNA detected and the MPN of CFU of *L. maculans* (R^2 = 0.94), as well as the number of leaf lesions on canola plants (R^2 = 0.95). Herdina and Roget (2000) conducted a soil bioassay, in which pre-germinated wheat seedlings were sown into infested soil and roots were rated for disease severity 4 weeks later (Herdina *et al.* 1997). They found a correlation of R^2 = 0.63 between the results of the bioassay and estimated *Ggt* DNA content in soil organic matter, which was estimated by hybridisation to the *Ggt* specific probe in a slot blot assay. A correlation of R^2 = 0.73 was obtained between the amount of *R. solani* DNA, also estimated by hybridisation to a probe specific for *R. solani* (AG-8) in a slot blot assay, and disease severity on the roots of wheat grown in soil which was artificially inoculated with *R. solani* (Siwek and Ophel-Keller 1997). Ophel-Keller *et al.* (1999) found a correlation of R^2 = 0.72 between the amount of *P. thornei* DNA, as estimated in an assay based on amplification of DNA using *P. thornei* primers in a PCR assay and microscopic counts of cereal cyst nematodes (CCN) in field soil samples. This assay was similar to that used for the *L. maculans* assay. The above-mentioned DNA-based assays for *Ggt*, *R. solani* and *P. thornei* are used in routine diagnostics by the RDTS, SARDI. The strong correlation of *L. maculans* DNA content and MPN of CFU and inoculum potential on canola leaves in the bioassay compares well with published assays and this observation suggests that the *L. maculans*-specific DNA assay could be applied to estimate the amount of DNA of this pathogen in soil.

Soil which has recently been cultivated with canola would be expected to contain canola stubble and the DNA assay should be sufficiently sensitive to detect *L. maculans* DNA in that stubble if the crop had been affected by blackleg. If *L. maculans* DNA was present in the field, Co1, which was sown to canola 3 years before sampling, it was below detection with the assay used here. *L. maculans* has been shown to survive for up to 4 years in some conditions in Australian soils (Bokor *et al.* 1975; McGee 1977; Barbetti and Khangura 1997).

However, in the present study, knowledge of the extent of blackleg disease in the previous crop of canola would be necessary to confirm if the fungus was unable to survive for 3 years in the field sampled. The variability in estimates of *L. maculans* DNA within field R9, which was sampled 1 year after canola was grown, was quite large and increased as the sampling intensity decreased. Results from this study suggested that a sampling intensity of at least 30 sub-samples (300 g in total) per 0.81 ha would be adequate to estimate the amount of *L. maculans* DNA in a field with a probability of 95%. Herdina and Roget (2000) found that only one sub-sample (500 g) per ha was adequate to estimate the amount of *Ggt* DNA in a field with a probability of 95%. The reason for the large difference between the minimum number of sub-samples for the *L. maculans* and *Ggt* DNA assays may be the much lower correlation between *Ggt* DNA and the bioassay ($R^2 = 0.63$) than between *L. maculans* and the bioassay in the present study ($R^2 = 0.95$). Reduction of variability in the estimation process by increasing sampling intensity will increase the precision of the DNA-based measurement.

As these results are based on sampling of only one field, it would be necessary to repeat the procedure for a number of fields with different soil types and in different climatic conditions to confirm the findings. The effect of sub-sample volume on the variability of the results was not assessed in this study. Herdina and Roget (2000) found that as sub-sample volume increased from 100 g to 500 g, the mean remained the same but variation between sub-samples decreased. However, based on the findings reported here, it would be appropriate to advise that when fields are sampled for testing for *L. maculans* DNA by the RDTS, SARDI, at least 30 sub-samples (300 g in total) per ha are required.

CHAPTER 8

PATHOGEN SURVIVAL

8.1 INTRODUCTION

L. maculans has been reported to persist on stubble for up to 5 years after a canola crop is harvested, and continue to produce viable ascospores over this time (Petrie 1995b; Baird *et al.* 1999). However, stubble decomposition is promoted by mild, wet conditions, such as those that occur in the United Kingdom, where the stubble can be completely decomposed within 2 years (West *et al.* 1999). It has been reported that *L. maculans* survives only on the canola stubble (Barbetti 1975; McGee 1977; Williams, 1992), but the minimum size of particles of organic matter that can sustain the fungus has not yet been determined.

In Australia, the persistence of stubble varies from 4 years in south western Australia to 1 year in south eastern Australia (Bokor *et al.* 1975; McGee 1977; Petrie 1978; Barbetti and Khangura 1997). This variation is mainly due to the difference in climatic conditions between the regions, namely hot dry summers in the south west and much milder conditions in the south east. The persistence of stubble in south central Australia has not been determined but is likely to be different from the other regions due to differences in climatic conditions.

Petrie (1995b) used ascospore liberation tunnels to study the long term survival and sporulation of *L. maculans* on stubble. The effect of burying stubble on pseudothecial development was investigated by Kharbanda and Ostashewski (1997), who counted pseudothecia on stubble. Isolations were made on selective media from stubble samples by Baird *et al.* (1999) to determine the relative longevity of the fungus on canola stubble. These traditional methods were labour intensive and time-consuming. The *L. maculans* specific

DNA assay, the validation of which was described in Chapter 7, was used to study the survival of the fungus on stubble and in soil, which is described in this chapter. Davidson *et al.* (2001) used a similar DNA assay, which was developed for the blackspot pathogens of field peas, to monitor survival of these pathogens in field soils.

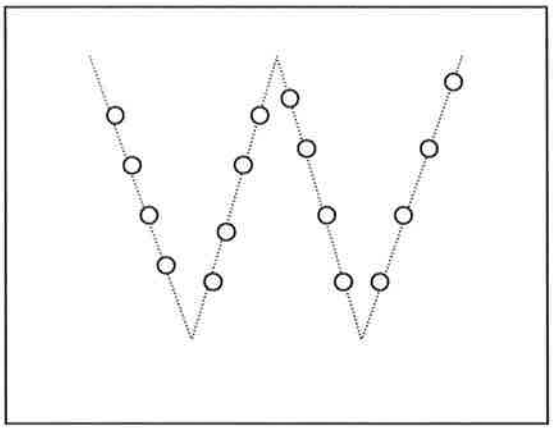
8.2 MATERIALS AND METHODS

8.2.1 Distribution of *L. maculans* in the soil profile

On April 19 2001, soil was collected from a property near Tarlee in South Australia. Eight fields were chosen based on canola rotation: Mu1 and Ki1, in which canola was grown in 2000; T2 and R9, in which canola was grown in 1999; E3, Ho1 and Co1, in which canola was grown in 1998; and A2, in which canola was grown in 1997 (Appendix 16). Co1 was initially incorrectly identified as having been sown to canola in 1997, but it was subsequently realised that it was actually sown to canola in 1998. One field, Ke2, in which canola had been grown in 2001, was sampled on April 16 2002.

Based on methods used by Davidson *et al.* (2001), three sub-samples of soil were collected from 16 randomly selected positions along a W transect over each entire field (Figure 8.1). Soil cores were removed using a soil sampler (15 cm long x 3 cm in diameter) and then separated into three depths (0-5 cm, 5-10 cm and 10-15 cm), combining soil from each depth together for each field into a single plastic bag. The soil samples (approximately 500 g each) were returned to the laboratory and stored in a cold room (3-4°C) overnight. The following day, soils were spread out in trays and dried in an oven (40°C) for a week, mixing every second day. Once dry, the amount of *L. maculans* DNA was then estimated from 400 g of each sample using the DNA-based assay (see section 7.2.2.4).

Figure 8.1: W transect pattern used to sample entire fields, with 16 sampling locations (O), from each of which three sub-samples were taken.



8.2.2 Survival of *L.* maculans on organic matter

8.2.2.1 Soil sampling

On April 16 2002, soil was collected from the same property near Tarlee in South Australia (sections 7.2.3 and 8.2.1). Eight fields were chosen based on canola rotation: W1 and Ke2, in which canola was grown in 2001; Mu1 and Ki1, in which canola was grown in 2000; T2 and R9, in which canola was grown in 1999; and Ho1 and Co1, in which canola was grown in 1998 (Appendix 16).

Four sub-samples were collected at random from 16 different positions along a W transect over each entire field (Figure 8.1). Soil cores were collected using a soil sampler (10 cm long x 2 cm in diameter, volume 31.43 cm^3), and all samples for each field were combined into a single plastic bag. The soil samples were returned to the laboratory, stored overnight and dried as described in section 8.2.1. Once dried, 500 g of each soil sample were processed to separate organic matter (OM) fractions, as follows.

8.2.2.2 Organic matter separation

Each soil sample was transferred into a plastic beaker (2 L) and topped up with tap water so that OM became suspended in the water. The water with suspended OM was poured through

a series of four laboratory sieves (Endecotts Ltd., England) of pore sizes 2 mm, 1.4 mm, 0.5 mm and 0.25 mm. This was repeated at least five times until all OM was removed from the water. The soil and OM particles smaller than 250 mm were discarded. The fractions of OM collected in each of the four sieves were rinsed and transferred on to filter paper discs (Whatmans No.1), which were placed in a porcelain funnel (Hirsch) connected to a vacuum source to remove excess water. OM fractions were left to dry overnight in the laboratory (approximately 22°C) and then put into paper bags for drying in a fan-forced oven (40°C) for 3 days. Once dry, each OM fraction for each field sample was weighed.

8.2.2.3 DNA extraction

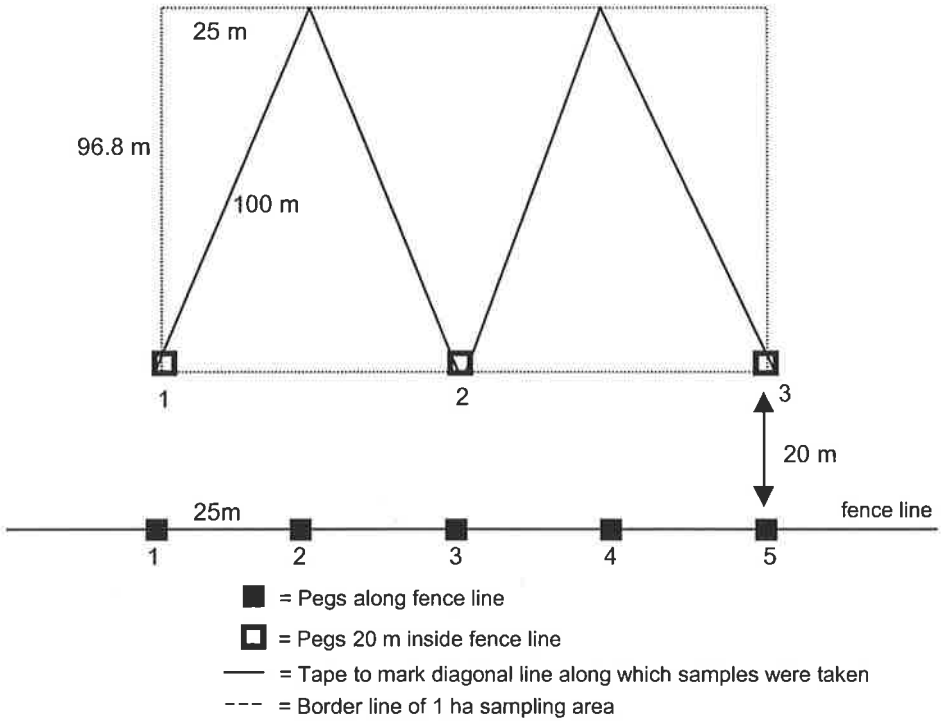
DNA was extracted from each fraction using the method described in section 7.2.2.4.

8.2.3 Effect of rotation

South Australian fields (Appendix 16) were surveyed over 3 years (2000-2002) in collaboration with J. Davidson, who was monitoring blackspot pathogens in soils of fields in which field peas were grown (Davidson *et al.* 2001). For each of the fields sampled, an area of 1 ha with uniform soil type and crop management was selected, headlands were avoided. Figure 8.2 shows the W transect pattern used to sample these fields. Five points along a fence line were pegged, 25 m apart, and then three pegs were placed 20 m inside the field perpendicular to the 1st, 3rd, and 5th pegs on the fence line, using an optic square. Two 100 m measuring tapes were attached to the 1st and 2nd pegs inside the field and extended until both tapes met at a point perpendicular to the 2nd peg along the fence line. This was repeated for the 2nd and 3rd pegs inside the field. Using a soil corer (10 cm long x 2 cm in diameter, volume 31.43 cm^3), 80 samples were taken at 5 m intervals along the diagonal lines (20 per line). Soil cores were combined for the whole field (approximately 2 kg), placed into plastic bags and returned to the laboratory for storage overnight in a cold room (3-4°C). The

following day, soil samples were placed on trays in an oven (40°C) and mixed every second day for the next week. Once dry, the amount of *L. maculans* DNA was then estimated from 400 g of each sample using the DNA-based assay (see section 7.2.2.4). Information on rotational history and soil type was collected from property managers (Appendix 16).

Figure 8.2: W transect pattern used to sample fields (Davidson *et al.* 2001).



8.3 RESULTS

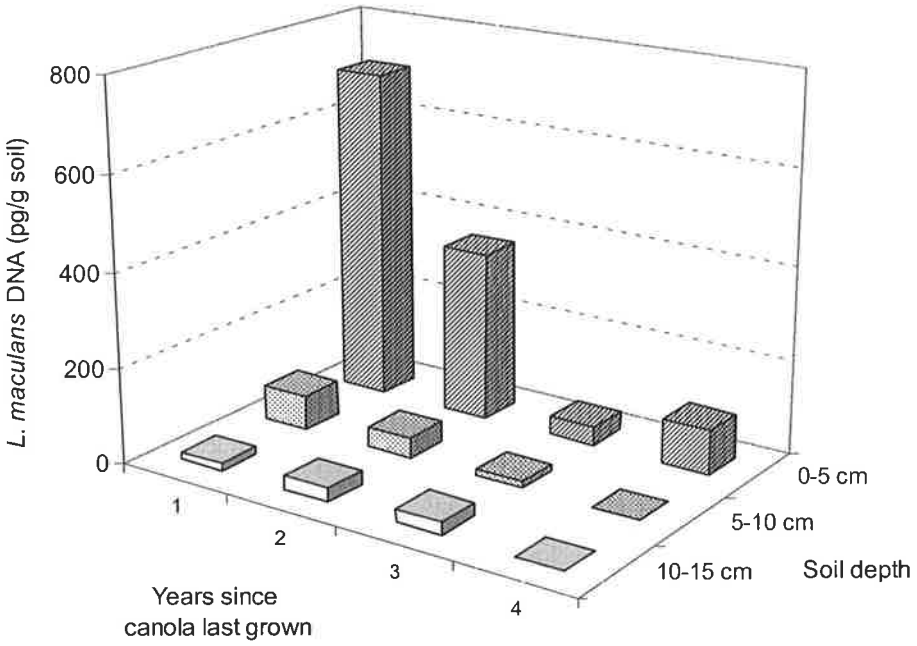
8.3.1 Distribution of *L. maculans* in the soil profile

The quantities of DNA (pg/g soil) for each soil sample, which were estimated by the RDTS, SARDI, are shown in Appendix 18. Analysis of variance revealed a significant difference ($P<0.05$) in the quantity of DNA between the top 5 cm of the soil profile and 5-15 cm below the surface (Table 8.1). There was no significant difference ($P>0.05$) between years since canola was last grown. Figure 8.3 shows the effect of years since canola was last grown and soil depth on the estimated quantity of *L. maculans* DNA.

Table 8.1: Analysis of variance table for *L. maculans* DNA in soil samples from three soil depths collected from fields in which canola was last grown between 1 and 4 years earlier.

Source	df	ss	ms	f	p
Field (A)	2	13904.50	6952.25	0.12	0.891
Year (B)	3	306848.00	102283.00	1.72	0.213
Depth (C)	2	600961.00	300481.00	5.04	0.024
B*C	6	501471.00	83578.50	1.40	0.285
A*B*C	13	774451.00	59573.20		
TOTAL	26	2197636.00			

Figure 8.3: Effect of years since canola last grown and soil depth on estimated quantity of *L. maculans* DNA. $LSD_{(0.05)}$ between soil depths averaged over years since canola grown = 215, $LSD_{(0.05)}$ between years since canola last grown averaged over the three soil depths = 248.



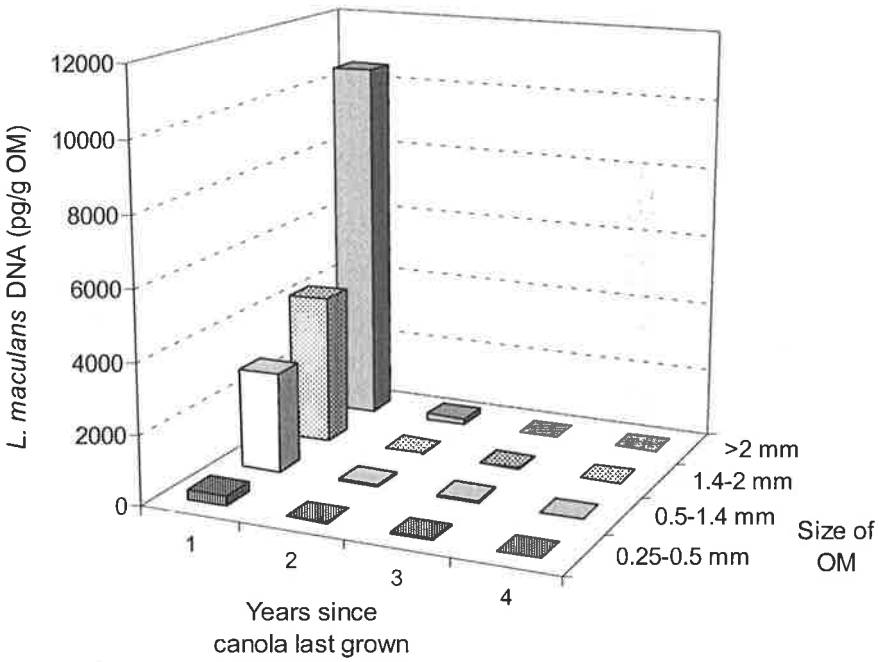
8.3.2 Survival of *L.* maculans on organic matter

The quantities of *L. maculans* DNA (pg/g OM) for each OM fraction of each field sample, which were estimated by the RDTS, SARDI, are shown in Appendix 19. Analysis of variance revealed a significant difference ($P<0.05$) in the mean estimated quantity of DNA between OM > 2 mm and OM < 2 mm as well as between OM < 0.5 mm and OM > 0.5 mm (Table 8.2). It also revealed that there was a significant difference ($P<0.05$) in the mean estimated quantity of DNA between OM of soil from fields in which canola was grown 1 year earlier and OM of soil from fields in which canola was grown 2-4 years earlier. Figure 8.4 shows the effect of years since canola was grown and OM fraction size on the estimated quantity of *L. maculans* DNA.

Table 8.2: Analysis of variance table for *L. maculans* DNA in four OM fractions of different particle sizes from soil samples collected from fields in which canola was last grown between 1 and 4 years earlier.

Source	df	ss	ms	f	p
Replication (A)	1	466470	466470	1.58	0.229
Year (B)	3	1.29E+08	4.31E+07	145.76	0.000
Size (C)	3	2.60E+07	8679077	29.33	0.000
B*C	9	7.59E+07	8433725	28.5	0.000
A*B*C	15	4438184	295879		
TOTAL	31	2.36E+08			

Figure 8.4: Effect of years since canola last grown and OM particle size on estimated quantity of *L. maculans* DNA. $LSD_{(0.05)}$ between OM sizes averaged over years since canola grown = 579, $LSD_{(0.05)}$ between years since canola last grown averaged over size of OM = 579.



8.3.3 Effect of rotation

The estimated quantities of *L. maculans* DNA in 98 field samples collected from 49 South Australian fields over 3 years are shown in Appendix 16. Values under 30 pg/g soil were considered to be 'background noise' and hence samples contained negligible amounts of *L. maculans* DNA. Fields in which canola was grown 1 year before sampling had a mean estimated *L. maculans* DNA quantity of 589 pg/g soil compared to 328 pg/g soil in fields in which canola had been grown 2 years earlier (Table 8.3). All other fields (3 -9 years since canola was last grown and fields in which canola had never been grown) had negligible *L.*

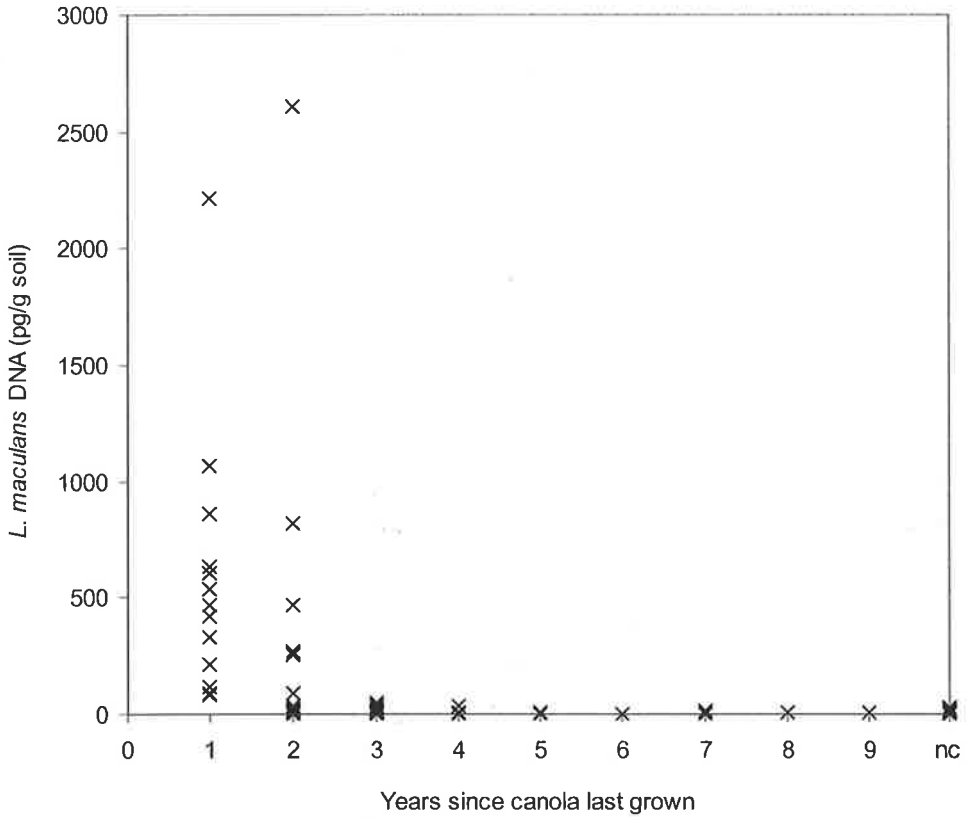
maculans DNA (mean < 15 pg/g soil). A one-way analysis of variance revealed that there was a significant difference ($P<0.05$) in estimated quantity of *L. maculans* DNA between years, but there was large variability within the fields in which canola had been grown 1 or 2 years before sampling. Of the 14 fields in which canola had been grown 2 years before sampling, seven contained less than 30 pg DNA /g soil, whereas all of the 14 fields in which canola had been grown in the previous year contained at least 86 pg DNA /g soil. Figure 8.5 shows the quantities of *L. maculans* DNA in soil samples from fields in which canola was grown 1-9 years before sampling as well as for fields which had never been sown with canola.

Table 8.3: Descriptive statistics of estimated quantities of *L. maculans* DNA in 98 soil samples collected from 49 fields around South Australia over 3 years (2000-2002) in collaboration with J. Davidson (SARDI).

Years since canola last grown	Number of samples	Mean (pg/g soil)	Standard error of mean	Min (pg/g soil)	Max (pg/g soil)	Standard deviation
1	14	589	147	86	2215	550
2	14	328	187	0	2610	698
3	9	14	5	0	47	18
4	5	10	6	0	34	14
5	2	3	3	0	6	4
6	1	2	n/a	2	2	n/a
7	2	6	6	0	12	9
8	1	9	n/a	9	9	n/a
9	1	6	n/a	6	6	n/a
N	49	3	1	0	28	6

N - fields not sown to canola in at least 10 years
n/a - not applicable

Figure 8.5: Quantity of *L. maculans* DNA, estimated by the Root Disease Testing Service, SARDI, in 98 soil samples collected from 49 fields around South Australia over 3 years (2000-2002) in collaboration with J. Davidson (SARDI). nc represents fields in which canola has never been grown.



8.4 DISCUSSION

Results from the RDTS revealed that the top 5 cm of the soil profile, which included stubble on the surface of the soil, yielded the most DNA of *L. maculans* and there was a significant decrease in the amount of *L. maculans* DNA detected between 5 and 15 cm below the surface. When OM was removed from the soil and fractionated, it was revealed that as OM size decreased, so too did the amount of *L. maculans* detected. As microbial activity is greater just below the surface of the soil where there is more moisture, stubble would be decomposed more rapidly (Turkington *et al.* 2000b). Species of *Trichoderma* naturally present in the soil are believed to exert some control of certain soil-borne pathogens (Papavizas 1985). Baird *et al.* (1999) suggested that *Trichoderma* spp. may be partially responsible for reducing the survival of *L. maculans*, amongst other pathogens, on buried canola stems, since the amount

of *L. maculans*, *Alternaria* and *Fusarium* spp. on stubble rapidly declined after burial and the amount of *Trichoderma* spp. increased. It is possible that *Trichoderma* spp. may have played a role in reducing the amount of *L. maculans* DNA detected at 5-15 cm below the soil surface in this study, but further experiments would need to be made to test this suggestion. Plant pathogens such as *R. solani* AG-4 have been reported to survive saprophytically in plant debris by utilizing cellulose as a carbon source (Baird *et al.* 1999). It is possible that the growth and maturation of pseudothecia depends on the presence of cellulose and pseudothecia may be decomposed by soil microorganisms, along with the stubble, although evidence of this is not yet available.

Management of canola stubble is an important factor in the reduction of inoculum of *L. maculans* for subsequent canola crops. Burying stubble by ploughing it in will cause it to decompose at a faster rate than if left on the surface and hence reduce the longevity of pseudothecia on the stubble (Blenis *et al.* 1999). Following the burial of stubble with shallow minimum tillage or direct seeding will prevent the stubble being returned to the surface (Gladders and Musa 1980). Furthermore, pseudothecia on buried stubble cannot release ascospores into the air (MacNish 1979). Raking and burning canola stubble is also an effective method of reducing inoculum (see section 2.8.3).

In the "soil depth experiment", the estimates of *L. maculans* DNA were not significantly different between fields in which canola had been grown in different years. The reason for this was the wide variation in amounts of DNA detected, which may have been caused by different weather conditions in each season or the use of canola cultivars with varying resistance to blackleg. However, in the "soil OM experiment" there was a significant difference between the amounts of *L. maculans* DNA detected in OM from fields in which canola had been grown in the previous year and fields in which canola had been grown 2 or

more years before sampling, suggesting that *L. maculans* did not survive more than 1 year after canola was grown.

The soil survey revealed that *L. maculans* DNA could be detected only in samples from fields in which canola was grown in the previous 2 years, and was not present in samples from fields in which canola was grown 3 or more years before sampling. Furthermore, all fields in which canola had been grown in the previous year yielded significant amounts of *L. maculans* DNA (i.e. > 30 pg/g soil), wheras only half of the fields in which canola had been grown 2 years before sampling yielded significant amounts of DNA of the pathogen. The fields surveyed represented much of the canola growing regions of South Australia, except for the south eastern region of the state. Therefore, in South Australia, the general recommendation should be that a rotation of 2-3 years is sufficient to prevent blackleg epidemics. However, canola should not be sown within 500 m of the previous year's stubble and preferably greater than 1500 m (Barbetti and Khangura 1999). Recent studies in Victoria have revealed that the majority of ascospores are released from 1-year-old stubble (Marcroft *et al.* 2001). Hence, a 2-year rotation is probably sufficient in Victoria and New South Wales, where conditions in summer are milder than in South Australia and there is more moisture to encourage rapid decomposition of the stubble. A 4-year rotation is currently recommended in Western Australia, where summers are usually hot and dry, which preserves stubble for longer periods (Barbetti and Khangura 1999). Due to the diverse climatic conditions which occur between and within states in Australia, it may be necessary to determine the most suitable rotation on a regional basis. This can now be achieved rapidly and efficiently with minimal costs using the *L. maculans* DNA assay.

The variation in amounts of *L. maculans* DNA that was observed in soil samples from fields in which canola had been grown 1 or 2 years earlier, may be due to a number of factors. The

density of the canola crops in each of the growing regions surveyed was different, which means that the amount of airborne inoculum from surrounding fields would have varied. Growers use a number of management strategies to control blackleg (section 2.8), and the extent to which they manage the disease will determine the level of inoculum remaining in the soil.

The DNA-based assay has reduced the time and effort needed for studies of pathogen survival and has increased the sensitivity and accuracy of results compared to traditional methods. Although it is a very useful tool for research, there is little potential of it being used as a commercial test for growers. As the major source of inoculum is air-borne ascospores (Hall 1992), which originate from surrounding fields in the district, testing the soil will not give the grower sufficient information to decide on the potential for blackleg infection.

CHAPTER 9

ROOT INFECTION

9.1 INTRODUCTION

Infection of all above-ground parts of the canola plant by *L. maculans* has been described in a recent review by West *et al.* (1999), however, there was no mention of root infection. It is generally understood that ascospores and pycnidiospores of *L. maculans* adhere to leaves and stems of plants and germinate to produce hyphae, which enter plants via stomata and wounds (Hammond and Lewis 1987a; Chen and Howlett 1996; Sosnowski *et al.* 2001b). There is no evidence of infection of canola roots by *L. maculans* directly from the soil, as occurs with *Rhizoctonia solani* (hypocotyl rot) in canola (Khangura *et al.* 1999) and in other plant-fungus interactions such as *M. pinodes* and *P. medicaginis* var. *pinodella* (blackspot) in field peas (Davidson *et al.* 1999) and *Ggt* (take-all) in wheat (Herdina and Roget 2000).

Towards the end of the growing season in 2000 there were reports from South Australia, Victoria and New South Wales of premature senescence of canola crops. In many cases, crops of resistant cultivars such as Dunkeld and Charlton had lodged with no external symptoms of blackleg. Plants were collected and sent to the SARDI Disease Diagnostic Service. The cause of this problem was investigated using laboratory procedures to examine the leaves, stems and roots of affected plants, both externally and internally, for symptoms of infection by *L. maculans*.

9.2 MATERIALS AND METHODS

9.2.1 Collection and diagnosis of samples

Canola plants (with roots intact) were sampled from 11 affected crops by farmers and agronomists (Table 9.1). Samples were collected when plants were at the flowering or

senescence stages (GS 4-5) and sent to the laboratory for diagnosis. Initially, plants were visually inspected in the laboratory for any disease symptoms.

Table 9.1: Samples collected from crops with premature senescence and lodging.

Sample number*	Nearest town to origin	Cultivar	Date collected
167/00	Bendigo, Victoria	n/a	13/10/00
189/00	Koppio, South Australia	n/a	31/10/00
197/00	Birchip, Victoria	Charlton	9/11/00
208/00	Bordertown, South Australia	Charlton	15/11/00
209/00	Bordertown, South Australia	Rainbow	15/11/00
211/00	Edillilie, South Australia	Charlton	20/11/00
212/00	Wanilla, South Australia	Dunkeld	20/11/00
222/00	Wonwandah, Victoria	PACY9043	1/12/00
4/01	Bribbaree, New South Wales	Ripper	8/11/00
5/01	Greenthorpe, New South Wales	Oscar	14/11/00
6/01	n/a, New South Wales	n/a	n/a

n/a – not available,
* entry number / year recorded

Samples of external and internal tissues were taken from both the stem and roots of the canola plants. They were surface sterilised, then sections (no more than 5 mm^2) were excised, plated on ¼ PDA and the cultures were incubated for 2 weeks as described in section 3.2.1.2. Cultures were identified based on keys by Punithalingam and Holliday (1972). Cultures identified as *L. maculans* were grown from single spores and stored as in section 3.2.1.2. Isolates were confirmed as *L. maculans* by the RDTS, using the DNA-based assay (section 7.2.2.4) and assigned to A or B-group (section 2.6).

To test for the presence of *Phytophthora* spp. and *Rhizoctonia solani*, samples of tissue (no more than 5 mm^2) were also placed directly on plates of $P_{10}VP^+$ selective medium and water agar (Appendix 3), respectively and incubated as in section 3.2.1.2. Cultures were identified based on Waterhouse and Waterston (1966) and Mordue (1974) for *Phytophthora* spp and *Rhizoctonia solani* respectively.

9.2.2 Pathogenicity testing

A glasshouse experiment was conducted to determine whether *L. maculans* could colonise the roots of canola via infection in the stem as well as enter the roots directly from the soil. The pathogenicity of isolate 208/00, taken from roots, was tested, along with the isolate 66/97, obtained previously from leaves, by inoculation of the roots, crown and leaves of canola plants. The experiment was arranged as a split plot design consisting of nine trays, each containing 12 punnets (550 ml). Each punnet was filled with potting soil (section 3.1.2.1) and sown with three seeds of cv. Hyola 42, later thinned to one plant. Plants at GS 2.3 were inoculated using pycnidiospore suspensions (10^6 spores/ml, section 3.2.1.4) of isolates 66/97 and 208/00. The control consisted of plants treated with sterile RO water with 0.05% Tween surfactant. The isolates and the control made up the three main plots. The suspensions of both isolates and water were applied to; 1. entire plants by spraying until run-off, 2. hypocotyls wounded with sterile toothpicks soaked in spore suspension or water and 3. lateral roots severed with surface-sterilised scissors and dipped into suspensions or water. The three methods of inoculation made up the sub-plots (four punnets per sub-plot). Trays were randomised within each of three replications and placed into humidity tents (section 6.2.1.3). After 4 days, the tents were opened and misting continued.

Twenty-two days after inoculation, two canola plants were removed from each treatment and soil removed from roots by rinsing under tap water. Roots were assessed for any symptoms. Isolations were made from root tissue using the methods described in section 9.2.1. Thirty-one days after inoculation, the plants remaining in the glasshouse were visually assessed for stem canker.

9.3 RESULTS

9.3.1 Collection and diagnosis of samples

Figure 9.1A shows canola plants (cv. Charlton) with premature senescence and lodging in a canola cultivar trial run by Mr Trent Potter, SARDI, near Bordertown, South Australia. Plants from affected crops were not cankered and only a few minor blackened lesions were present on the stem and roots (Figure 9.1B). Dissection of affected plants revealed extensive necrosis in both the pith of the stem and the tip of the taproot in plants which had little or no external symptoms (Figure 9.1C). In many cases, roots with blackened areas had no new root growth (Figure 9.1D). In one case, a distinct black band was seen around the circumference of the taproot approximately 10 mm below the crown (Figure 9.2A). Cross sections show that no necrotic tissue was present between the band and the stem (Figure 9.2B) and internal necrosis existed beneath the band (Figure 9.2C).

All cultures isolated from necrotic tissue, internal and external, were identified as *L. maculans* based on cultural characteristics and morphology (Punithalingham and Holliday 1972). All isolates were confirmed as A-group *L. maculans* using the DNA-based assay (RDTS, SARDI).

9.3.2 Pathogenicity testing

Plants inoculated, via wounded hypocotyls and lateral roots, with either isolate of *L. maculans* developed the distinctive blackening on roots as seen in field samples, within 22 days of inoculation (Table 9.2; Figure 9.2D). Plants inoculated by spraying with spore suspensions of either isolate developed leaf lesions, but not blackened roots, within 22 days of inoculation. Control plants treated with sterile RO water, using all three methods, had no symptoms on leaves or roots. Isolations from all blackened tissue samples yielded *L. maculans*. Thirty-one days after inoculation, assessment of plants that remained in the glasshouse revealed that stem

cankers developed on plants inoculated with both isolates via the hypocotyls (Figure 9.2D) and lateral roots (Figure 9.2E), however root symptoms were more severe on the plants inoculated via the roots. Stem cankers did not develop on plants inoculated by spraying with spore suspension or on plants treated with sterile RO water, using all three methods.

Table 9.2: Root infection and stem canker development on plants of cv. Hyola 42 when inoculated with two *L. maculans* isolates (208/00 and 66/97) either by 1. spraying entire plants with spore suspensions, 2. wounding hypocotyls with a toothpick soaked in spore suspension or 3. severing lateral roots with scissors and dipping into spore suspensions. Control plants were treated with sterile water.

Days after inoculation		22		31
Isolate	Inoculation method	Blackened roots	*L. maculans* isolated*	Stem canker
control	1. spray	✖	✖	✖
	2. hypocotyl	✖	✖	✖
	3. root	✖	✖	✖
66/97	1. spray	✖	✖	✖
	2. hypocotyl	✓	✓	✓
	3. root	✓	✓	✓
208/00	1. spray	✖	✖	✖
	2. hypocotyl	✓	✓	✓
	3. root	✓	✓	✓

* *L. maculans* isolated from roots
Three replications per treatment

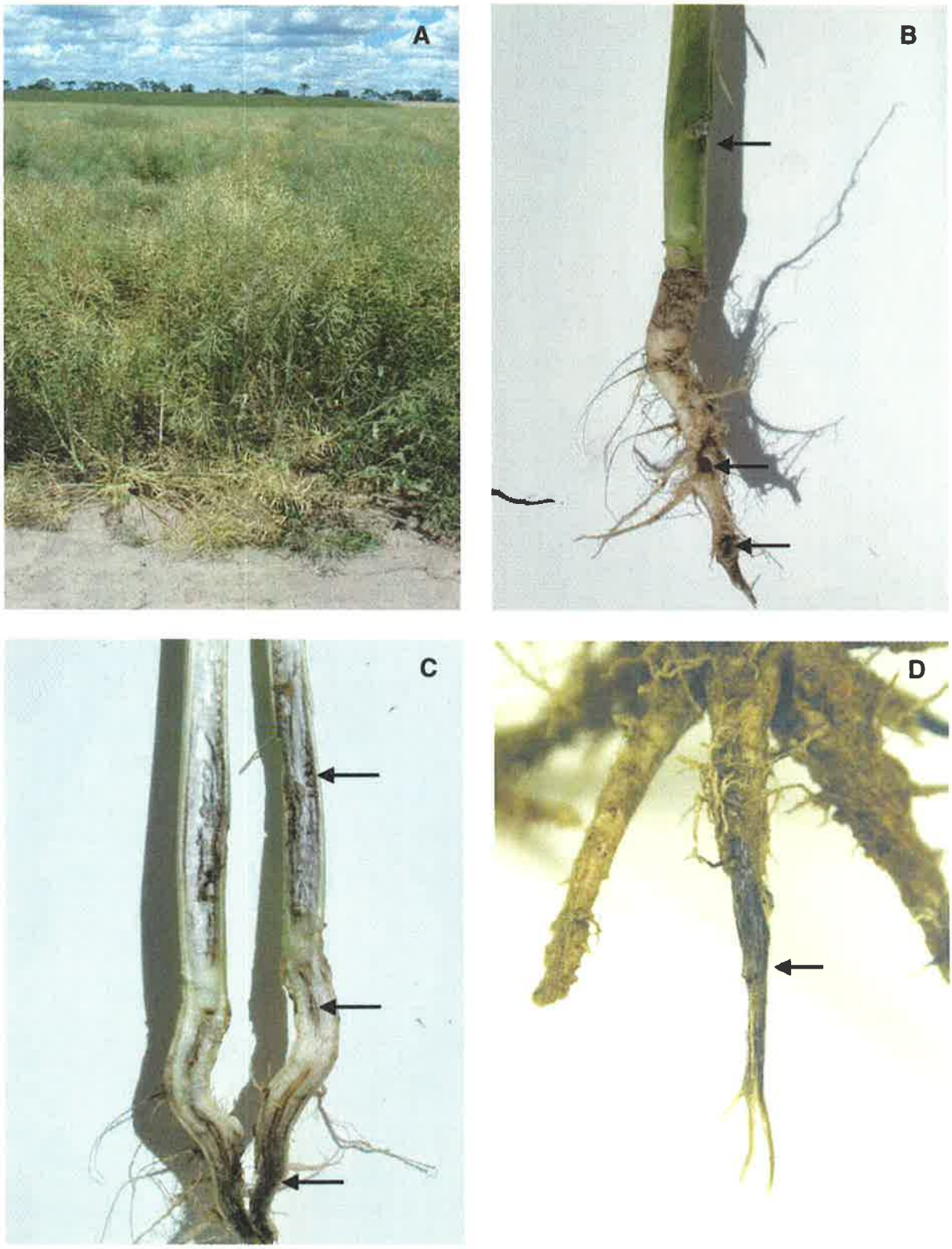


Figure 9.1: A. Canola plants (cv. Charlton) near Bordertown, in south eastern South Australia, which showed premature senescence and lodging. B. On closer inspection, one of the Charlton plants appeared to have minor lesions on the stem and root. C. The plant was dissected, revealing extensive internal necrosis in the pith of the stem and the tip of the taproot (isolate 208/00 taken from here), as well as some necrosis in the upper stem. D. Roots of cv. Dunkeld, collected from near Wanilla, on the Eyre Peninsula in South Australia, with blackening on a lateral root (isolate 212/00 taken from here) and no new root growth from that area. Arrows point to areas with blackened tissue.

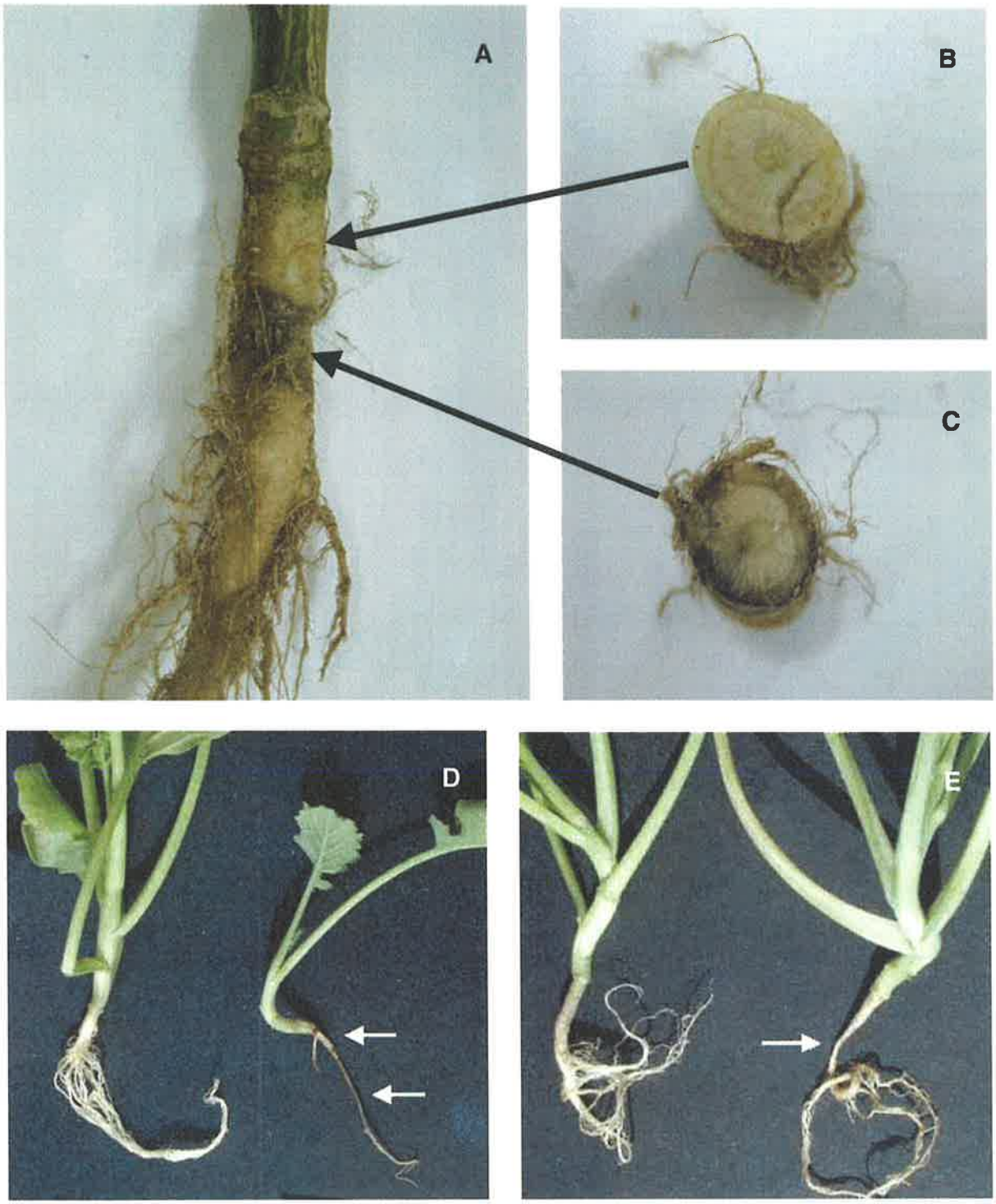


Figure 9.2: A. Tap root of a canola plant collected near Koppio, on the Eyre Peninsula in South Australia, with a distinct black band. B. Cross-section of the root between the black band and the crown, revealing no discolouration. Arrow shows where section was taken. C. Cross-section of the root within the black band, exposing black tissue (isolate 189/00 taken from here). Arrow shows where section was taken. D. Thirty one days after root inoculation of cv. Hyola 42 with *L. maculans* isolate 208/00 (right) showing a crown canker and blackening of the taproot with no new lateral root growth, compared to the control plant with healthy roots (left). E. Thirty one days after hypocotyl inoculation of cv. Hyola 42 with *L. maculans* isolate 208/00 (right) showing a crown canker and restriction of the taproot compared to the control plant with healthy roots (left). White arrows point to areas with blackened tissue.

9.4 DISCUSSION

Although external symptoms were not obvious on plants showing premature senescence or lodging, they were found to have severe internal infection of the stem and roots by *L. maculans*. Such infection causes degradation of the vascular tissue, which contains the xylem and phloem vessels responsible for water and nutrient transport between the roots, leaves and pods (Salisbury and Ross 1992). This causes senescence of the plant and, if it occurs before pods have developed, seed size and number will be reduced along with oil content and quality. The degradation of pith and cortex tissue also weakens the base of the stem, causing the plant to lodge. In some samples infected tissue stretched from the stem down into the roots, suggesting that the fungus grew from the stem to colonise the roots. However, in some cases, there was a distinct area of tissue free from *L. maculans* between areas of root and stem infection, which implied that the fungus had entered directly from the soil into the roots. The current national blackleg rating system is based on survival of canola plants (Marcroft 2001). Since the fungus can reduce yield without killing plants or developing external symptoms, it is therefore important to consider the internal symptoms by cutting stems and rating for staining. This is currently being done in Western Australia, in addition to survival counts (Khangura *et al.* 2002), since internal staining has been related to yield (Khangura and Barbetti 2001).

In the glasshouse experiment, application of pycnidiospores to wounds made to the roots and hypocotyls of canola seedlings, led to severe infection of the roots and eventually crown canker 4-5 weeks after inoculation. Infection via the roots caused more severe root symptoms than via the hypocotyl, possibly because more wounds were produced by severing roots compared to a puncture of the hypocotyl with a toothpick. In the field, roots and hypocotyls can be wounded in a number of ways, such as damage by insects, nematodes, other soil fungi and vehicle tyres. As ascospore and pycnidiospore infection are very similar (section 2.4.3),

it is likely that hyphae from ascospores could also enter through root and hypocotyl wounds. Ascospores may be released from stubble under the soil surface and, since they can survive for up to 6 weeks (Paul and Rawlinson 1992, cited by West *et al.* 1999), they have the potential to infect canola roots which grow alongside them. Root infection should be considered a part of the disease cycle, as it could have ramifications for management of the disease.

Spraying inoculum over the entire plants did not result in any root disease symptoms 4-5 weeks after inoculation. This may have been because the duration of the experiment was not long enough for the fungus to reach the roots via the stems. Since it appeared that *L. maculans* infection could spread from the stem down into the roots, it could be assumed that under optimal conditions for infection (section 2.4.3), plants which were inoculated by spraying with pycnidiospores would eventually develop root symptoms.

This work has been published (Sosnowski *et al.* 2001a) and is the first official record of symptoms of blackleg on canola roots in Australia. However, there is anecdotal evidence that such symptoms have been observed in the past (D. Ballinger, personal communication 2001; M. Barbetti, personal communication 2001). For effective management of root infection, further investigation is required. First, the incidence of this type of infection should be assessed by a survey and then the effects of environmental conditions on incidence and severity of root infection need to be determined as well as the pathway of infection through the roots.

CHAPTER 10

GENERAL DISCUSSION

This study has revealed new information about the interactions between *L. maculans* and canola, and the mechanisms by which canola can resist pathogen invasion. The epidemiology of the fungus and factors which affect the severity of disease have been examined and a preliminary model has been developed to attempt to predict stem canker by consideration of the weather conditions before and after the crop is sown. A new method of studying pathogen survival was developed by means of validating a DNA-based assay, and the longevity of the pathogen in South Australian soils was shown to be 1 or 2 years. Finally, *L. maculans* was discovered in the roots of canola, and entry via the stem and directly through the roots from soil was demonstrated.

Histochemical staining of canola plants inoculated with *L. maculans* in the glasshouse revealed that lignin and suberin occurred in the epidermal cell walls and stomatal guard cells of both resistant and susceptible cultivars although the concentration of lignin and suberin appeared to be lower in the susceptible cultivar. This supported the results of field and glasshouse trials, in which the incidence and severity of leaf lesions did not differ between the cultivars examined, except for Hyola 60. It is likely that production of lignin and suberin in the epidermal cell walls and stomatal guard cells has little impact on infection by *L. maculans*. Leaf infection was observed on every plant assessed in the field trials, except for those of cv. Hyola 60. Given that screening for blackleg resistance has been based largely on the reaction of canola stems to infection (Thurling and Venn 1977; Newman and Bailey 1987; Cargeeg and Thurling 1980; Sjödin and Glimelius 1988; Marcroft *et al.* 2002), it is not surprising that reaction at the leaf surface does not play a role in the resistance of most current cultivars to blackleg.

Infection of cotyledons and leaves on Hyola 60, a cultivar released for commercial use in Australia in 2001, was very low compared to all other cultivars tested. The incidence of stem infection on Hyola 60 was also significantly lower than on the other cultivars, and this has been attributed in part to the reduction in infection of leaves and petioles. Similar observations were made by Crouch *et al.* (1994), who transferred genes conferring leaf resistance from a wild accession of *B. rapa* var. *sylvestris* from Sicily into *B. napus*. SEM images of the leaf surface of inoculated Hyola 60 plants, showed that spore germination was reduced and hyphal growth restricted compared to the susceptible cultivar, Q2, which suggested that a biochemical mechanism was acting at the early stage of infection. The use of biochemical assays, such as thin layer chromatography, may enable the location and isolation of one or more anti-fungal chemicals associated with resistance in cv. Hyola 60. Such an anti-fungal compound could then be characterised, for example by mass spectroscopy, and may provide a selection tool for breeders or even have potential as a fungicide.

Scanning electron micrographs of petioles of inoculated seedlings revealed hyphae of *L. maculans* were located in the xylem vessels and cortical cells adjacent to the xylem in both resistant (cv. Scoop) and susceptible (cv. Westar) cultivars of canola. In the stem, hyphae were observed only in the xylem and cortex of the susceptible cultivar. This led to the conclusion that resistance in cv. Scoop was conferred by an interaction with the pathogen at the stem-petiole junction. Hammond and Lewis (1986b) found that in the resistant cv. Primor, lignification of the parenchyma cells of the cortex and pith surrounding the vascular tissue was associated with the limitation of infection. Therefore, it is likely that a similar mechanism occurs in cv. Scoop but further histochemical studies of the stem-petiole junction are required to confirm this suggestion. In both field and glasshouse trials, senescence of leaves was prevalent on all cultivars, so accumulation of lignin, and possibly suberin, in the

xylem vessels and parenchyma cells in the stem/petiole junction of resistant plants may act to slow hyphal growth until senescence occurs.

Several cultivars were assessed in the field and in the glasshouse for their response to infection by *L. maculans*. The incidence of stem canker on the susceptible cv. Q2 was similar in the field and in the glasshouse, suggesting that this cultivar has little or no resistance to blackleg. However, it appeared that all the other cultivars tested, except Hyola 60, developed less leaf infection and stem canker in the glasshouse than on average in the four field trials. Pycnidiospores were used to inoculate plants in the glasshouse and the majority of natural inoculum in the field comprised ascospores, except for the barley grain inoculum which comprised mycelia and pycnidiospores. So it may be that ascospores are more efficient at germinating and penetrating leaves, leading to systemic infection, which confirms findings by Wood and Barbetti (1977b). Furthermore, plants in the field may have been stressed due to nutrient and water deficiencies along with the presence of other plant pathogens. Optimal conditions for plant growth were provided in the glasshouse, so it is possible that cultivars other than Q2 and Hyola 60 were able to inhibit colonisation of the stem by *L. maculans* more effectively than in the field. In the glasshouse, approximately 14% of plants of cv. Hyola 60 developed stem canker whereas, in the field, stem canker did not occur on this cultivar. This suggested that the ability of Hyola 60 to resist *L. maculans* was not affected by stress from factors which could not be controlled in the field.

This study has shown that resistance is an important factor in reducing the potential of a blackleg epidemic and that factors such as rain, temperature and wind can determine the extent of the epidemic. Other factors, such as inoculum concentration, plant maturity during infection and other pathogens can also have an effect on an epidemic, but can be controlled by using appropriate management strategies.

Canola cultivars currently grown in Australia, with the exception of Hyola 60, have variable resistance to stem infection by *L. maculans* but are susceptible to leaf infection. Airborne inoculum, in the form of ascospores released from pseudothecia on canola stubble, is greatest during the first 2 months of the growing season, when plants are at the cotyledon and early leaf growth stages and most vulnerable to infection. Plants inoculated between the cotyledon stage (GS 1) and the first true leaf stage (GS 2.5) had a high incidence of stem canker, whereas plants that were inoculated at the elongation stage (GS 3.1) or later, developed very little stem infection. This confirmed reports by McGee and Emmett (1977) and McGee and Petrie (1979) that canola plants were most vulnerable to stem infection when inoculation occurred during early leaf growth.

A new procedure has been developed to study the survival of *L. maculans*. A number of nucleic acid techniques have been developed previously for the identification of *L. maculans* from pure fungal cultures and infected canola leaves (refer to section 2.5.2), but not stubble. This study has used an improved semi-selective medium and bioassay to validate a DNA-based assay for use in assessing the amount of *L. maculans* DNA in stubble and soil samples.

L. maculans was shown to survive for up to 2 years on stubble in soil samples from fields in which canola had previously been grown. Future research might involve the examination of effect of cultivar, size of stubble fragments, weather conditions and soil type on the decomposition of stubble in soil by conducting experiments in the glasshouse and controlled environment chamber. Survival of the fungus in controlled conditions could be assessed using the DNA-based assay and plant bioassays, enabling confirmation of results from field samples in this study.

Further development of this DNA assay procedure could provide a rapid and efficient means of testing seed for infection by *L. maculans*. Previous studies showed that contaminated seed was not generally a major source of inoculum but that it could introduce the fungus to new areas (Wood and Barbetti 1977a; McGee 1977; Gabrielson 1983; Hall *et al.* 1996). However, in view of the genetic diversity of the pathogen and the variation which exists between countries (West *et al.* 2001), it is important to investigate whether isolates of the fungus in Australia are able to cause epidemics on canola cultivars through infected seed. The availability of the quantitative DNA assay would allow such studies to be carried out more accurately and efficiently than in the past.

Data from field trials in this study were used to produce a preliminary model to predict the incidence of stem canker, based on a number of weather factors as well as cultivar selection. The model also predicts the potential yield loss from stem canker in some cultivars. This model needs to be validated and developed further by conducting a series of trials in different regions and incorporating additional information such as sowing time, fungicide treatment, rotation, stubble management and distance from last year's crop. A similar model for blackspot in field peas is currently being developed (J. Davidson, personal communication, 2002). In the pathogen survival studies, the effect of rotation and stubble management was examined and, if combined with results from trials in Victoria and Western Australia on the effect of distance from last year's crop (Marcroft *et al.* 2001; Khangura and Barbetti 2001), a more accurate predictive model could be developed. A model, currently being developed in Western Australia (Salam *et al.* 2002) for predicting maturation of pseudothecia and the timing and extent of ascospore release, is being further developed using data from this study as well as other epidemiology trials in progress around Australia. Therefore, there is potential to consolidate these models and data from trials in all canola-growing states of Australia to develop a national decision support system for the management of blackleg in canola. It is envisaged that a computer-based package could be developed and made available to growers

and agronomists, as has been developed by the Oilseed Pathology Group at Rothamstead Research – Integrated Approach to Crop Research (IACR) in the United Kingdom (www3.res.bbsrc.ac.uk/leafspot/, 2002) for the prediction of light leaf spot of oilseed rape.

Protection of plants from leaf infection by *L. maculans* from germination to the elongation stage, effectively mimicking the anti-fungal mechanism in leaves of the cv. Hyola 60, may allow a reduction in colonisation of the stem and, hence, lower the incidence and severity of stem canker. The only fungicide registered for the control of blackleg in Australia is Impact® (flutriafol), which is applied in a mixture with fertiliser (Ballinger *et al.* 1988; Khangura and Barbetti 2001) and provides disease control during seedling establishment. Benomyl was tested as a foliar fungicide to control stem canker in Australia without success (Brown *et al.* 1976). Other foliar fungicides, such as triazoles (Rempel and Hall 1995), propiconazole (Kharbanda *et al.* 1999) and difenoconazole or flusilazole with carbendazim (Gladders *et al.* 1998 cited by West *et al.* 2001; Sun *et al.* 2001), have been tested in Europe and Canada with mixed results. Timing of application may be crucial to the efficacy of fungicides, as ascospore infection is generally greatest when rainfall, temperature and wind speed increase. The information from this study on the weather conditions which are conducive to infection and through the model being developed in Western Australia to predict the timing of ascospore dispersal, the efficiency of these fungicides may be improved by applying them only when ascospores have been released and conditions are conducive for infection. There are also many foliar fungicides registered for controlling pathogens in other crops in Australia, such as chlorothalonil and mancozeb for the control of ascochyta blight in chickpeas and blackspot in field peas (Anonymous 2002b). Hence, there is potential for the development of strategic application of foliar fungicides to control blackleg in Australia.

Infection of the roots of canola by *L. maculans* has been reported for the first time and there was evidence of direct infection through the roots in field-grown plants. A glasshouse

experiment revealed that the fungus was able to enter the roots directly from the soil via wounds, although the inoculum used in this experiment is unlikely to occur naturally in soil, as pycnidiospores do not survive alone in the soil for longer than a few months (Gabrielson 1983). Therefore, it is necessary to determine whether wounded roots can be infected in soil containing naturally infested stubble. Ascospores, which are released from pseudothecia on the stubble under the soil surface, could come into contact with canola roots and enter through wounds caused by insects, nematodes, other soil fungi and vehicle tyres. An experiment, involving inoculations in controlled conditions, could be designed to test this hypothesis.

Further research on root infection may involve the pathway of infection by *L. maculans* within the root. Using the scanning and transmission electron microscopes, it may be possible to determine the pathway of hyphal growth within the cortex and vascular tissue of the root. Factors such as cultivar and weather may have an effect on the extent of root infection and should be examined along with the effect of root infection on yield.

In conclusion, this study has provided new information on the interactions between *L. maculans* and canola and highlighted the importance of this disease to the canola industry. Although this pathogen can devastate canola crops, management strategies which have been developed through research, such as this study, may be improved to achieve effective control. However, the continued success of the canola industry requires more research, such as that suggested above, and new sources of resistance to combat disease caused by this genetically diverse fungus.

APPENDICES

Appendix 1: Canola cultivars available in 2002, including year of release, type, maturity, blackleg rating, oil and protein content (Potter and Stanley 2002).

Cultivar	Year	Type	Maturity	Blackleg*	Oil#	Protein
Ag-Emblem	1999	Conventional[a]	Early-mid	7	Moderate	Moderate
Ag-Outback	2001	Conventional	Early	7	Moderate	Moderate
ATR-Beacon	2002	Triazine[b] tolerant	Mid	6	High	
ATR-Grace	2001	Triazine tolerant	Mid-late	6.5 P	Moderate	High
ATR-Hyden	2001	Triazine tolerant	Mid	6	Moderate	Moderate
Charlton	1998	Conventional	Mid-late	6	Very high	High
Clancy	1997	Triazine tolerant	Mid	6	Moderate	Moderate
Drum	1997	Triazine tolerant	Early-mid	5.5	Moderate	High
Dunkeld	1993	Conventional	Mid-late	6	High	High
Georgie	1999	Conventional	Early-mid	5.5	Moderate	Very high
Hyola 60	2001	Hybrid	Mid	9	High	Very high
Insignia	1999	Conventional	Mid-late	6	Very high	High
Karoo	1996	Triazine tolerant	Early-mid	3.5	Moderate	Moderate
Monty	1997	Conventional	Early	4.5	Moderate	Moderate
Mystic	1998	Conventional	Early-mid	6	Moderate	Moderate
Oscar	1992	Conventional	Mid	6	Moderate	Moderate
Pinnacle	1997	Triazine tolerant	Mid-late	5.5	Moderate	Mod/high
Purler	1999	Conventional	Mid-late	6.5	Highest	Highest
Rainbow	1993	Conventional	Mid	6	Moderate	Moderate
Ripper	1999	Conventional	Mid	6.5	Very high	Very high
Scoop	1997	Conventional	Mid	6	High	High
Surpass 300TT	2001	Triazine tolerant	Very early	4.5	Moderate	Moderate
Surpass 400	1999	Conventional	Early	9	High	Very high
Surpass 402CL	2001	Clearfield[c]	Early-mid	8.0 P	Moderate	Moderate
Surpass 501TT	2001	Triazine tolerant	Early-mid	na	High	na
Surpass 600	1999	Conventional	Mid	6	Very high	High
Surpass 600TT	1999	Triazine tolerant	Mid-late	5.5	Mod/high	High
Surpass 603CL	2001	Clearfield	Mid	8.0 P	Very high	Very high
Trooper	1999	Conventional	Mid	6	High	Moderate
44C71	1999	Clearfield	Early-mid	5	Moderate	Very high
44C73	2001	Clearfield	Early-mid	5.5 P	Moderate	na
45C75	2001	Clearfield	Early-mid	6.5 P	Moderate	na
46C03	1999	Conventional	Mid	6	Moderate	Moderate
46C72	1999	Clearfield	Mid	5.5	High	High
46C74	2001	Clearfield	Mid	6.0 P	Moderate	na
47C02	1998	Conventional	Mid-late	5.5	Moderate	Moderate

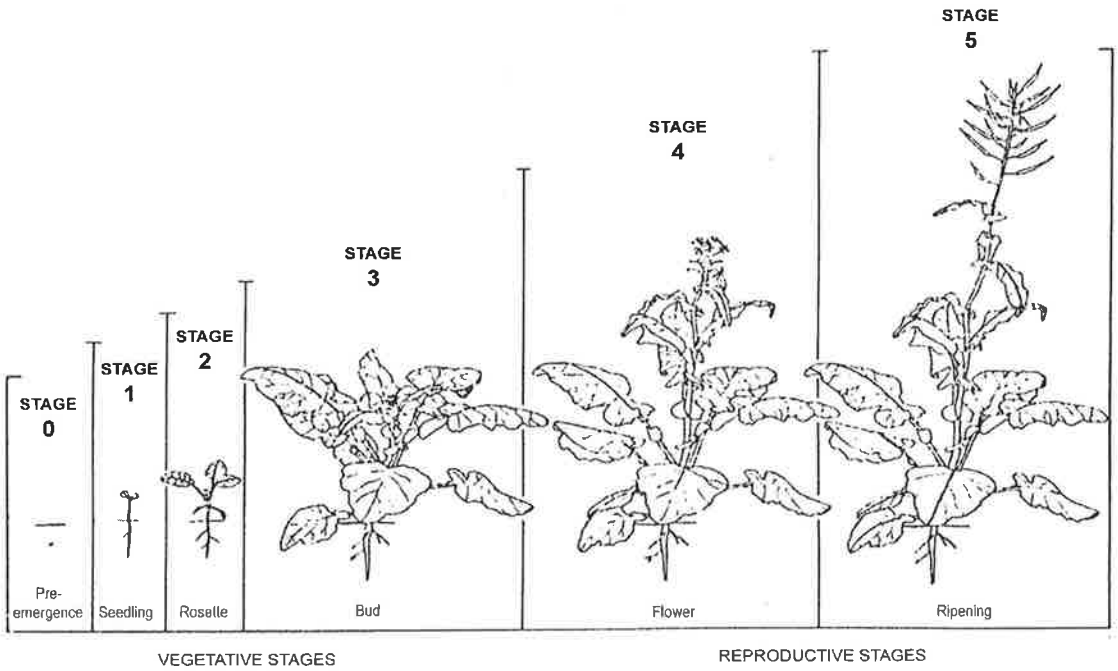
* Blackleg survival ratings published by the Canola Association of Australia, from nursery trials around Australia by Departments of Agriculture and private breeding companies. Results statistically analysed. 1 = susceptible, 9 = resistant. P – Provisional, cultivars not tested over six sites and 2 years.

Oil content of cultivars can vary considerably from year to year and site to site within a year. Oil content of a cultivar varies little relative to other varieties of similar maturity grown under the same conditions.

[a]Conventional – cultivar bred conventionally
[b]Triazine – herbicide
[c]Clearfield – cultivars resistant to imidazolinone herbicides
na – not available

Appendix 2: Growth-stage key for *Brassica napus* (Harper and Berkenkamp 1975).

GS 0	Pre-emergence
GS 1	Seedling
GS 2	Rosette
	GS 2.1 First true leaf expanded
	GS 2.2 Second true leaf expanded
	(add 0.1 for each additional leaf)
GS 3	Bud
	GS 3.1 Inflorescence visible at centre of rosette
	GS 3.2 Inflorescence raised above level of rosette
	GS 3.3 Lower buds yellowing
GS 4	Flower
	GS 4.1 First flower open
	GS 4.2 Many flowers open, lower pods elongating
	GS 4.3 Lower pods starting to fill
	GS 4.4 Flowering complete, seeds enlarging in lower pods
GS 5	Ripening
	GS 5.1 Seeds in lower pods full size, translucent
	GS 5.2 Seeds in lower pods green
	GS 5.3 Seeds in lower pods green-brown mottled
	GS 5.4 Seeds in lower pods brown
	GS 5.5 Seeds in all pods brown, plants senescent



Appendix 3: Media for culturing fungal isolates.

Water agar	
Bacto-agar (Difco)	20 g
RO (reverse osmosis) water	1000 ml
1% Streptomycin sulphate (Sigma)	10 ml
Potato Dextrose Agar (PDA)	
Potato dextrose agar (Oxoid)	39 g
RO water	1000 ml
1% Streptomycin sulphate (Sigma)	10 ml
Quarter Strength Potato Dextrose Agar (¼ PDA)	
Potato dextrose agar (Oxoid)	10 g
Bacto-agar (Difco)	10 g
RO water	1000 ml
1% Streptomycin sulphate (Sigma)	10 ml
$P_{10}VP^+$ selective medium (Tsao and Ocana 1969)	
Corn meal agar – CMA (Difco)	17 g
RO water	1000 ml
Anti-microbial agents	
Pimafucin (Sigma)	0.4 ml
Vancomycin hydrochloride (Sigma)	0.3 g
0.5% Hymexazole	10 ml
0.25% Pentachloronitrobenzene – PCNB (Sigma)	10 ml
0.5% Streptomycin sulphate (Sigma)	10 ml

Culture media were autoclaved at 121°C for 20 min and, in a sterile laminar flow unit, 1% streptomycin sulphate antibiotic (1 g/100 ml sterile RO water) and anti-microbial agents were added to molten media, once cooled to approx 50°C. Agar media were dispensed into 9-cm diameter Petri dishes, approx. 15 ml per plate, and allowed to cool and solidify.

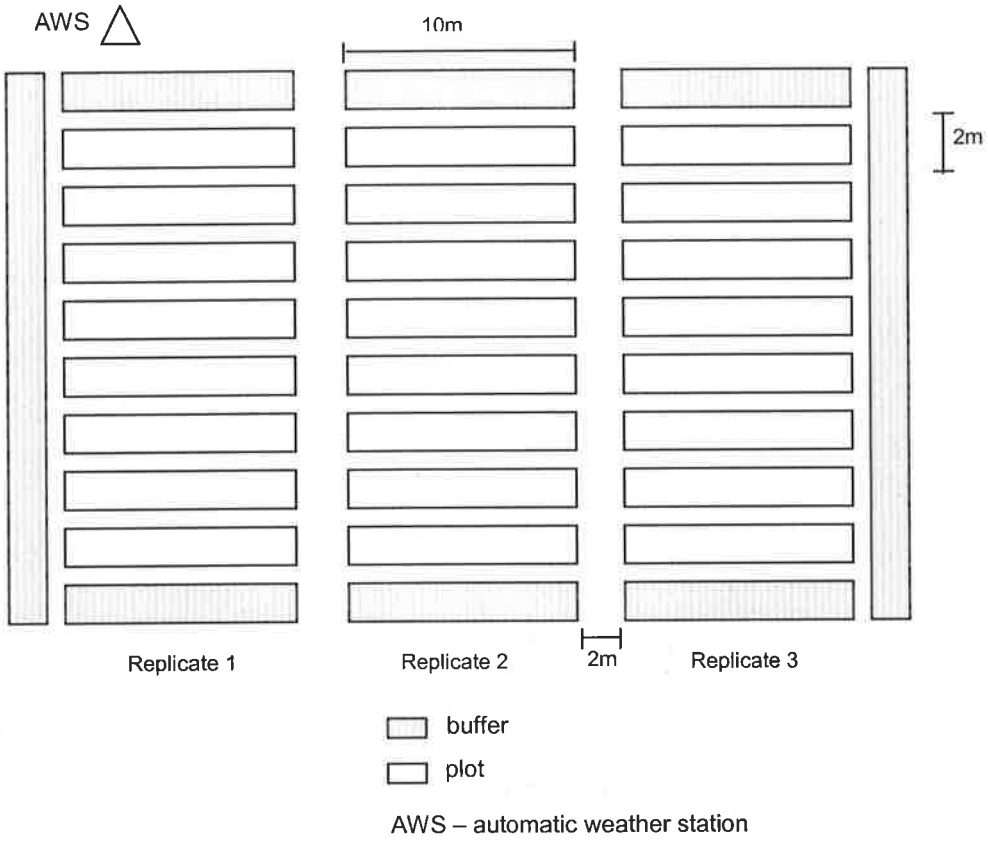
Appendix 4: Routine fixative for preserving plant tissue samples in storage.

Gluteraldehyde fixative	
Gluteraldehyde (EM grade, 25% solution)	2.5 ml
Paraformaldehyde	4 g
Sucrose	4 g
PBS buffer	100 ml

Dissolve 4 g paraformaldehyde in buffer by heating to 60-65°C (in a fume hood) and add 4 g sucrose. Allow to cool. Add 2.5 ml gluteraldehyde (EM grade, 25% solution) and make up to 100 ml with PBS buffer.

Phosphate buffered saline (PBS) – (0.01M phosphate, 0.15M NaCl, 0.003M KCl)	
Sodium chloride	0.8 g
Potassium dihydrogen orthophosphate	0.02 g
Di-sodium hydrogen orthophosphate	0.14 g
Potassium chloride	0.02 g
RO water	100 ml
Adjust to pH 7.4	

Appendix 5: Experimental design for epidemiological field trials.



Appendix 6: Information about field trials at Kingsford RS (Research Station), 7 km north of Gawler, South Australia and Charlick RS, 9 km south of Strathalbyn, South Australia.

Field	Kingsford RS 2000 Centre middle paddock	Kingsford RS 2001 Tank paddock	Charlick RS 2000 Paddock 6W	Charlick RS 2001 Paddock S
GPS coordinates	S 34°32.662' E 138°47.131'	S 34°33.303' E 138°47.130'	S 35°20.155' E 138°52.621'	S 35°19.966' E 138°52.476'
Elevation	510 ft	454 ft	200 ft	200 ft
Soil type	Red brown earth medium clay / medium clay	Red brown earth sandy clay loam/ medium clay	Red brown earth	Red brown earth
Rotational history	1996 – cereal trials 1997 – barley 1998 – pasture 1999 – pasture	1997 – cereal trials 1998 – barley 1999 – pasture 2000 – pasture	1996 – barley 1997 – legume plots 1998 – wheat 1999 – wheat (Janz)	1997 – cereal plots 1998 – barley 1999 – canola 2000 – wheat (Janz)
Cultivation	Two workings with a CASE wideline cultivator	Two workings with a CASE wideline cultivator	1st Fieldspan cultivator 2nd Combine presowing	1st Offset disc 2nd Combine presowing
Pre-sowing Treflan DAP (18:20) Endosulfan	 May 10 (1 L/ha) May 10 (90 kg/ha) -	 May 30 (1 L/ha) May 30 (90 kg/ha) May 30 (2.1 L/ha)	 May 8 (2 L/ha) May 8 (100 kg/ha) May 9 (1 L/ha)	 May 24 (2 L/ha) May 24 (100 kg/ha) May 25 (1 L/ha)
Sowing date	May 11 17:10 N:P (50kg)	May 31 17:10 N:P (120kg)	May 10 17:10 N:P (50kg)	May 25 17:10 N:P (120kg)
Sowing rate	5 kg/ha	5 kg/ha	5 kg/ha	5 kg/ha
Post-sowing	May 14 Endosulfan (1L/ha) July 11 Lontrel (300ml/ha) July 13 Select (250ml/ha)	June 5 Endosulfan (1L/ha) July 28 Lontrel (300ml/ha) Verdict (100ml/ha)	June 22 Mesurol snail bait	July 19 Mesurol snail bait July 27 Verdict (100ml/ha)
Windrowing date	Nov 10	Nov 21	-	-
Harvesting date	Nov 15	Dec 6	-	Nov 30

DAP® = diammonium phosphate (nitrogen:phosphate)
Treflan® = 480 g/L trifluralin
Endosulfan® = 350 g/L endosulfan
Verdict® = 130g/L haloxyfop
Select® = 240g/L clethodim
Lontrel® = 300g/L clopyralid

Trials at Kingsford RS were prepared by Peter McCormack, Farm Manager. Sowing, maintenance and harvesting carried out by the SARDI Field Crop Evaluation Program.
Trials at Charlick RS were prepared and maintained by Michael Elleway, Farm Manager. Sowing and harvesting carried out by the SARDI Field Crop Evaluation Program.

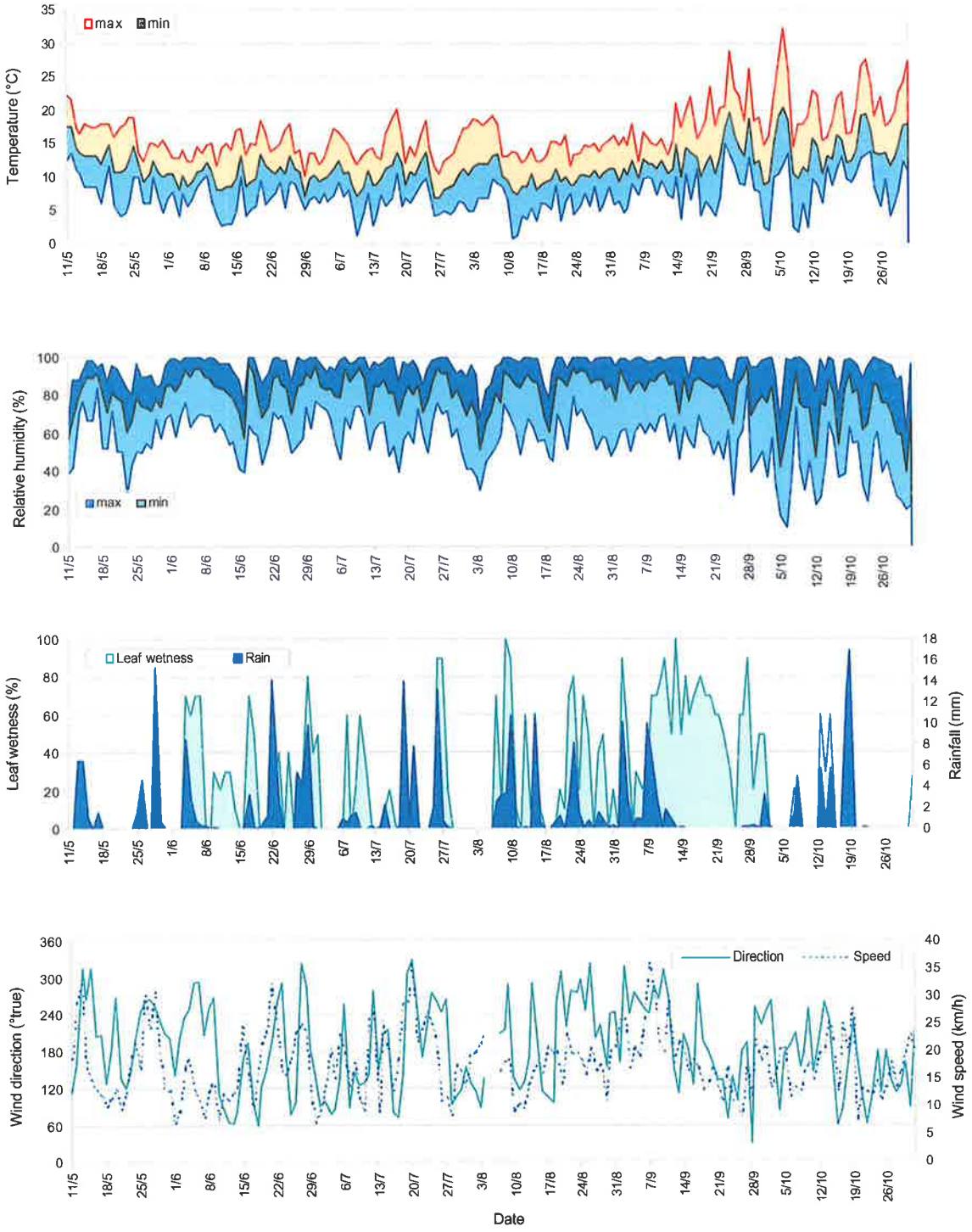
Appendix 7: Rating scales for blackleg disease assessment in field and glasshouse trials.

Rating	Cotyledon infection
0	No lesion on cotyledon
1	Lesion on cotyledon
	Leaf and petiole infection
0	No leaf and petiole infection
1	<5% of leaf area infected, no petiole infection
2	>5% of leaf area infected, no petiole infection
3	<50% of petiole length infected
4	>50% of petiole length infected
	Stem infection*
0	No infection
1	Stem lesion <10mm
2	Stem lesion 11-20mm
3	Stem lesion 21-30mm
4	Stem lesion >30mm
5	Stem canker
6	Plant lodged
7	Plant dead

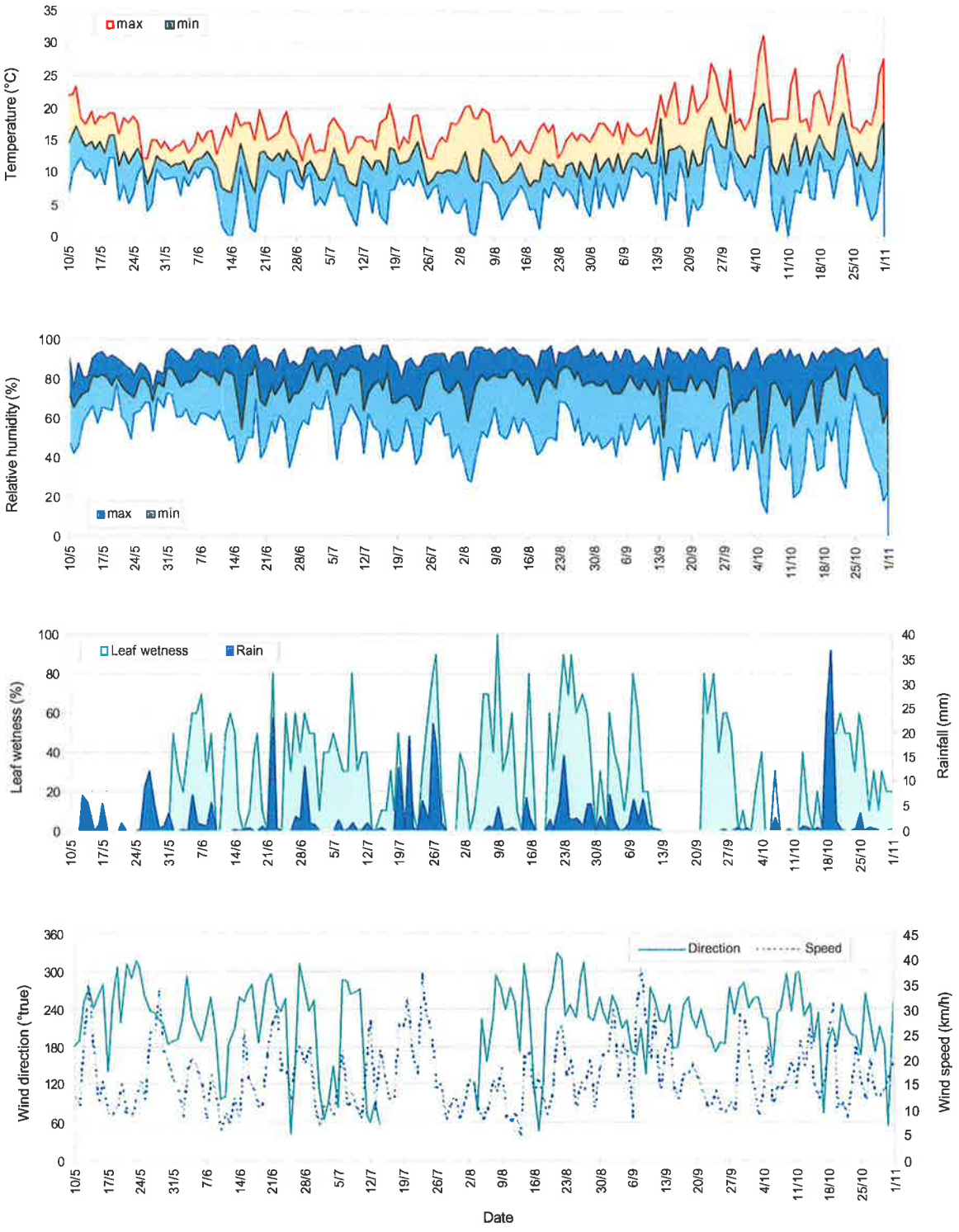
*Stem infection scale derived from Hanacziwskyj & Drysdale (1984) and Kutcher (1990) cited by van den Berg *et al.* (1993)

Other scales developed in this project.

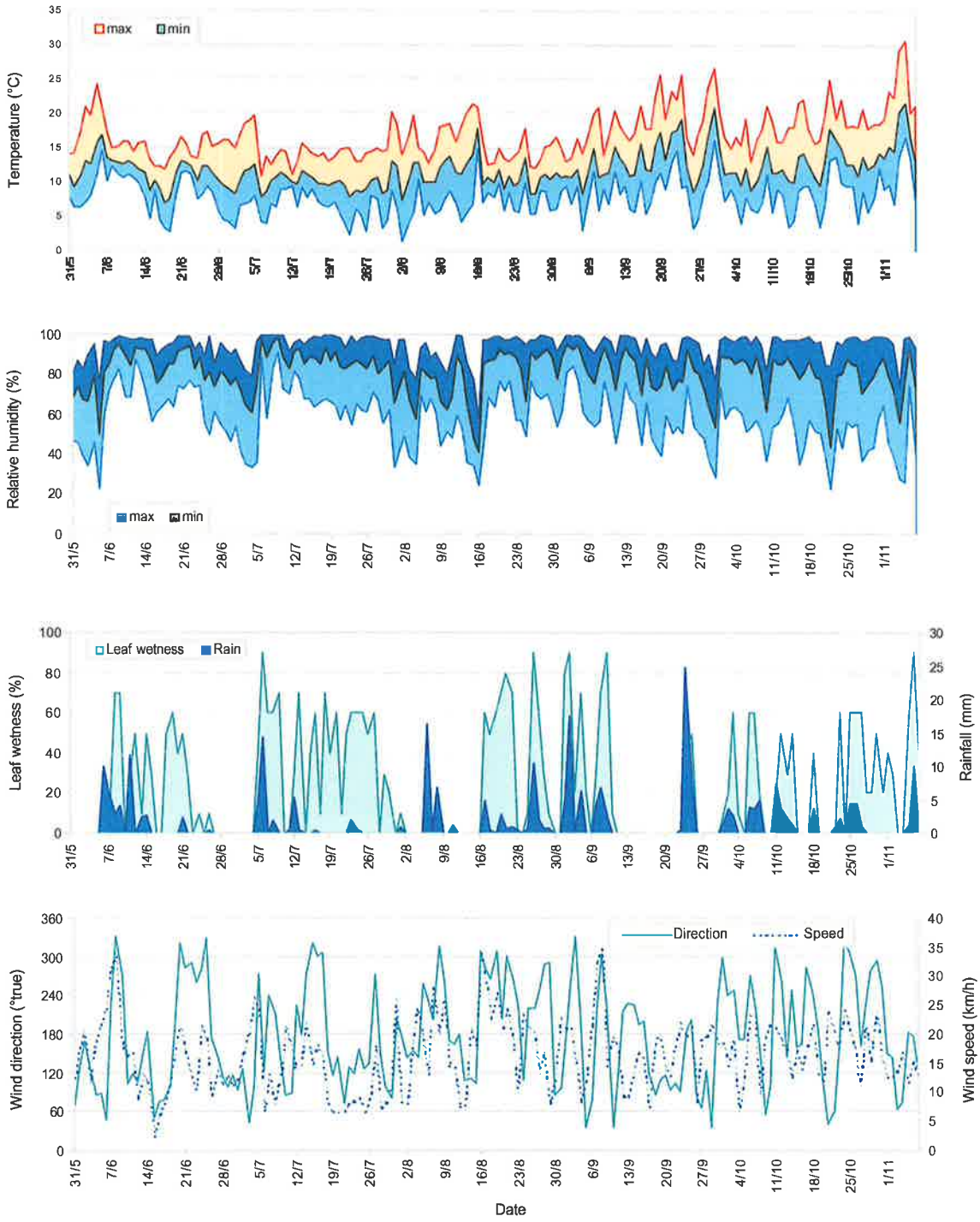
Appendix 8: Weather data collected by an automatic weather station (AWS) positioned next to the Kingsford trial (temperature, relative humidity, rainfall and leaf wetness) and from the Bureau of Meteorology, Roseworthy AWS (wind) during the 2000 season. For both temperature and relative humidity, minimum (min), mean and maximum (max) daily reading are shown. For leaf wetness, rainfall, wind speed and wind direction, daily means are shown.



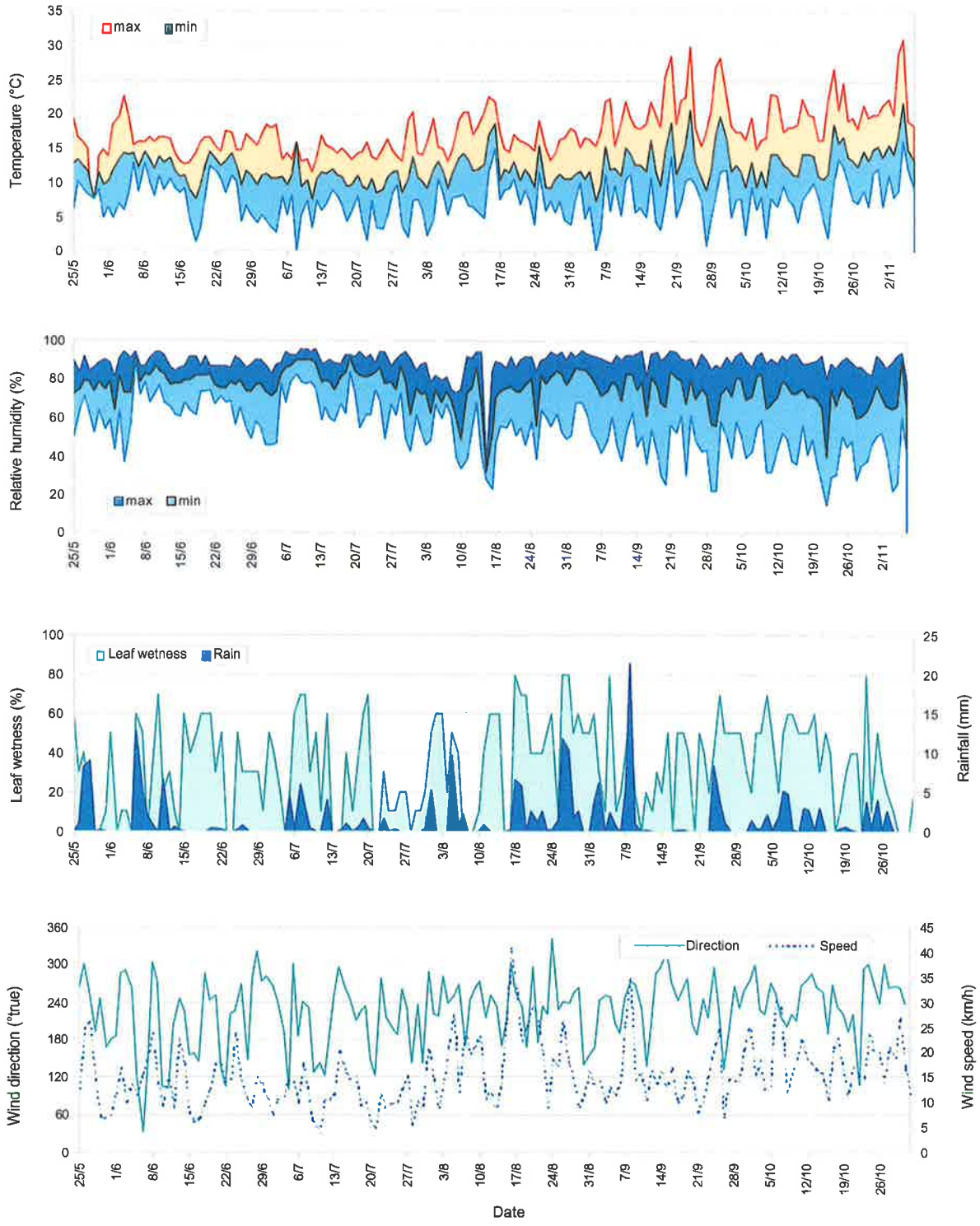
Appendix 9: Weather data collected by an automatic weather station (AWS) positioned next to the Charlick trial (temperature, relative humidity, rainfall and leaf wetness) and from the Bureau of Meteorology, Strathalbyn AWS (wind) during the 2000 season. For both temperature and relative humidity, minimum (min), mean and maximum (max) daily reading are shown. For leaf wetness, rainfall, wind speed and wind direction, daily means are shown.



Appendix 10: Weather data collected by an automatic weather station (AWS) positioned next to the Kingsford trial (temperature, relative humidity, rainfall and leaf wetness) and from the Bureau of Meteorology, Roseworthy AWS (wind) during the 2001 season. For both temperature and relative humidity, minimum (min), mean and maximum (max) daily reading are shown. For leaf wetness, rainfall, wind speed and wind direction, daily means are shown.



Appendix 11: Weather data collected by an automatic weather station (AWS) positioned next to the Charlick trial (temperature, relative humidity, rainfall and leaf wetness) and from the Bureau of Meteorology, Strathalbyn AWS (wind) during the 2001 season. For both temperature and relative humidity, minimum (min), mean and maximum (max) daily reading are shown. For leaf wetness, rainfall, wind speed and wind direction, daily means are shown.



Appendix 12: Leaf infection on spore trap plants exposed for 1-week periods throughout two growing seasons (2000 and 2001) at the Kingsford and Charlick trial sites.

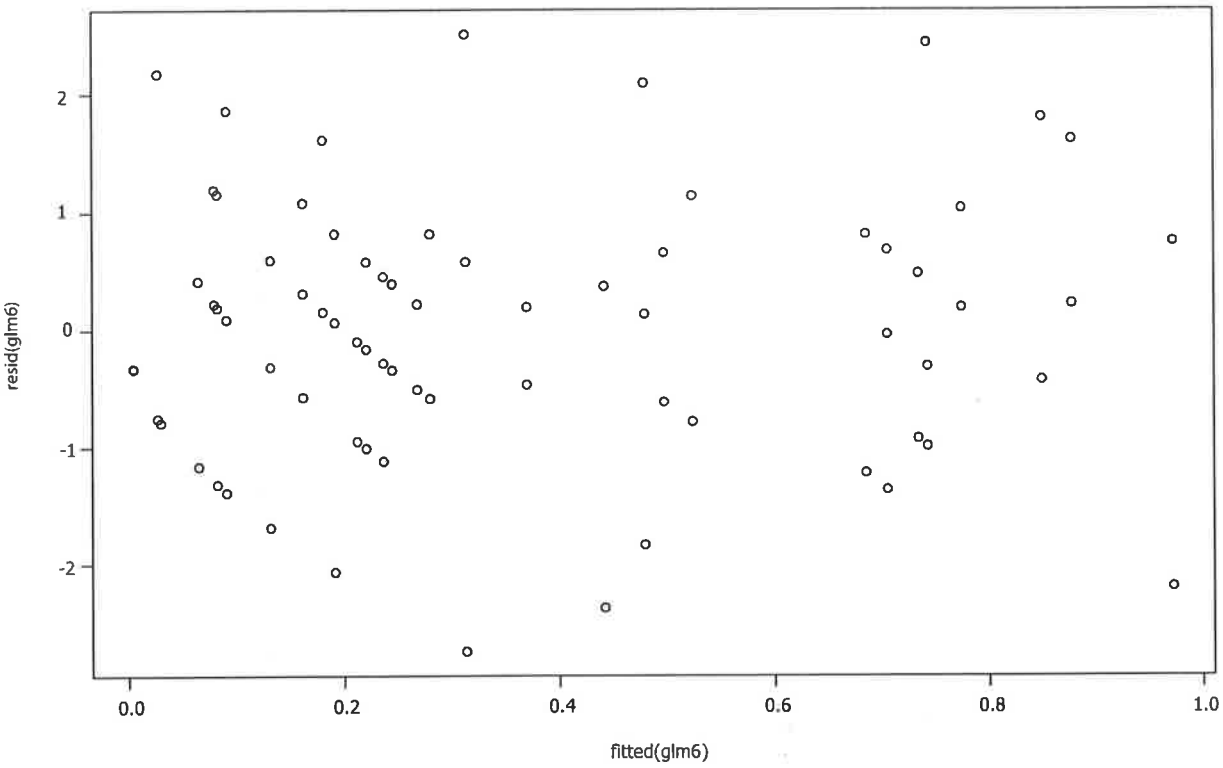
Kingsford 2000		Charlick 2000		Kingsford 2001		Charlick 2001	
Period exposed (day/month)	Mean no. lesions / plant	Period exposed (day/month)	Mean no. lesions / plant	Period exposed (day/month)	Mean no. lesions / plant	Period exposed (day/month)	Mean no. lesions / plant
				16/5 - 22/5	0	16/5 - 23/5	0
				23/5 - 29/5	2.3	24/5 - 30/5	1.4
31/5 - 6/6	14.1	31/5 - 7/6	18.2	30/5 - 5/6	1.4	31/5 - 6/6	6.6
7/6 - 14/6	2.9	8/6 - 15/6	12.4	6/6 - 12/6	2.5	7/6 - 13/6	2.9
15/6 - 21/6	10.7	16/6 - 22/6	24.4	13/6 - 19/6	11	14/6 - 20/6	2.6
22/6 - 28/6	37.3	23/6 - 29/6	22.2	20/6 - 26/6	6	21/6 - 27/6	0.9
29/6 - 4/7	3.7	30/6 - 5/7	16.2	27/6 - 3/7	0.2	28/6 - 3/7	0.3
5/7 - 11/7	15.3	6/7 - 13/7	12.8	4/7 - 10/7	5.1	4/7 - 11/7	4.4
12/7 - 19/7	14.7	14/7 - 21/7	15.3	11/7 - 17/7	14	12/7 - 19/7	6.8
20/7 - 26/7	21.4	22/7 - 28/7	25.8	18/7 - 24/7	1.3	20/7 - 25/7	2.9
27/7 - 1/8	2.8	29/7 - 2/8	3	25/7 - 1/8	1.9	26/7 - 2/8	0.3
2/8 - 9/8	12.6	3/8-10/8	19.3	2/8 - 8/8	32.9	3/8 - 9/8	4.6
10/8 - 15/8	3.5	11/8 - 16/8	4.3	9/8 - 14/8	0.2	10/8 - 15/8	0.3
16/8 - 23/8	4.3	17/8 - 24/8	3.2	15/8 - 22/8	18.2	16/8 - 23/8	3.8
24/8 - 30/8	1.9	25/8 - 31/8	6.4	23/8 - 28/8	5.4	24/8 - 29/8	1.5
31/8 - 5/9	3.4	1/9 - 6/9	2.7	29/8 - 4/9	15.9	30/8 - 5/9	14.9
6/9 - 12/9	4.7	7/9 - 13/9	3.7	5/9 - 11/9	5.7	6/9 - 12/9	1.1
13/9 - 20/9	0	14/9 - 21/9	0	12/9 - 18/9	0.1	13/9 - 19/9	0.5
21/9 - 26/9	0	22/9 - 27/9	0	19/9 - 25/9	0.4	20/9 - 26/9	0.2
27/9 - 3/10	0	28/9 - 4/10	0	26/9 - 3/10	0.2	27/9 - 3/10	0
4/10 - 10/10	0	5/10 - 11/10	0	4/10 - 9/10	0.2	4/10 - 10/10	0.3
11/10 - 19/10	0.1	12/10 - 20/10	0	10/10 - 16/10	0.4	11/10 - 17/10	0
20/10 - 26/10	0	21/10 - 26/10	0	17/10 - 23/10	0	18/10 - 24/10	0
				24/10 - 30/10	0	25/10 - 31/10	0
				31/10 - 6/11	0	1/11 - 7/11	0
				7/11 - 13/11	0	8/11 - 14/11	0
Total	153.4	Total	189.9	Total	125.3	Total	56.3

Appendix 13: Yield data from trials in 2000 and 2001 at Kingsford and selected disease incidence and severity ratings at different times throughout the season.

Cultivar	Year	Rep	Yield (t/ha)	CI June (%)	SI Aug (%)	SC Aug (%)	SS Nov (1-7)	SI Nov (%)	SC Nov (%)
Dunkeld	2000	1	2.88	30	0	0	1.8	60	0
Dunkeld	2000	2	2.78	50	0	0	2.9	80	0
Dunkeld	2000	3	2.61	70	10	0	2.9	70	20
Dunkeld	2001	1	2.72	60	0	10	5.2	100	80
Dunkeld	2001	2	2.49	100	0	10	5.4	100	80
Dunkeld	2001	3	2.61	90	0	10	3.8	80	50
Hyola 42	2000	1	2.50	20	30	10	4.3	100	30
Hyola 42	2000	2	2.93	60	20	0	2.7	70	20
Hyola 42	2000	3	2.75	60	0	10	2.5	100	20
Hyola 42	2001	1	1.53	70	0	40	5.5	90	80
Hyola 42	2001	2	0.46	70	0	50	6.3	100	80
Hyola 42	2001	3	1.64	50	30	50	6.4	100	90
Hyola 60	2001	1	2.44	0	0	0	0.1	10	0
Hyola 60	2001	2	3.06	0	0	0	0	0	0
Hyola 60	2001	3	3.04	10	0	0	0	0	0
Karoo	2000	1	1.98	20	30	0	3.3	80	30
Karoo	2000	2	1.81	20	10	10	3.3	80	20
Karoo	2000	3	1.79	20	40	0	4	90	30
Karoo	2001	1	0.94	70	20	40	0.5	100	100
Karoo	2001	2	1.32	60	10	10	0.5	100	80
Karoo	2001	3	1.06	80	30	50	0.6	100	80
Monty	2000	1	2.96	40	40	0	4.4	100	20
Monty	2000	2	3.03	70	30	10	5.1	100	40
Monty	2000	3	2.56	20	30	10	3.7	90	20
Monty	2001	1	1.58	80	10	10	0.7	100	80
Monty	2001	2	2.14	80	20	20	0.4	100	80
Monty	2001	3	2.17	70	10	20	0.1	100	60
Mystic	2000	1	3.39	30	30	0	2.8	70	0
Mystic	2000	2	2.89	10	10	0	1.8	50	0
Mystic	2000	3	3.13	40	0	0	3.1	80	0
Mystic	2001	1	2.04	90	0	10	4.5	100	40
Mystic	2001	2	2.11	90	0	0	3.9	80	40
Mystic	2001	3	2.86	90	10	20	5	100	60
Pinnacle	2000	1	2.48	50	0	0	1.1	30	0
Pinnacle	2000	2	2.12	20	0	10	1.8	50	10
Pinnacle	2000	3	2.29	20	0	0	1.4	40	0
Pinnacle	2001	1	2.65	70	10	10	2.7	70	10
Pinnacle	2001	2	2.38	90	0	0	4.1	90	30
Pinnacle	2001	3	2.28	100	20	20	3.1	70	20
Q2	2000	1	1.29	60	20	30	6.1	100	80
Q2	2000	2	1.69	20	0	10	5.3	100	50
Q2	2000	3	1.81	50	0	0	5.1	90	70
Q2	2001	1	0.99	90	20	50	6.6	100	100
Q2	2001	2	1.04	60	20	30	5.7	100	80
Q2	2001	3	0.74	80	40	50	6.4	100	100
Scoop	2000	1	3.16	10	10	0	2.4	70	0
Scoop	2000	2	2.81	10	30	10	2.9	70	10
Scoop	2000	3	2.61	30	10	0	4	90	20
Scoop	2001	1	2.86	90	0	0	0	90	50
Scoop	2001	2	2.43	80	0	10	0.1	90	80
Scoop	2001	3	1.98	90	0	0	0.1	70	20

CI – cotyledon infection, SI – stem infection, SC – stem canker, SS – stem severity (rating scale 1-7)

Appendix 14: Residual versus fitted plot for the generalised linear model to predict stem canker incidence on a range of canola cultivars.



Appendix 15: Table used to ascertain the most probable number of colony forming units in soil samples by determining the number (m) of *L. maculans* spores estimated from the positive plate counts with two or four replications (r) and two-fold dilution steps (s) (adapted from Somasegaran and Hoben 1985).

Positive plates		Dilution step(s)			
r=4	r=2	s=10	s=8	s=6	s=4
40	20	520			
39		520			
38	19	520			
37		370			
36	18	290			
35		220			
34	17	180			
33		140			
32	16	120	130		
31		95	130		
30	15	78	130		
29		65	93		
28	14	54	72		
27		45	55		
26	13	37	45		
25		31	35		
24	12	26	29	33	
23		21	24	33	
22	11	18	19	33	
21		15	16	23	
20	10	13	13	18	
19		11	11	14	
18	9	8.9	9.3	11	
17		7.4	7.7	8.9	
16	8	6.3	6.4	7.4	
15		5.2	5.4	6.0	
14	7	4.4	4.6	4.9	8.3
13		3.7	3.8	4.1	5.9
12	6	3.2	3.2	3.4	4.6
11		2.6	2.6	2.7	3.4
10	5	2.2	2.2	2.3	2.8
9		1.8	1.9	1.9	2.2
8	4	1.5	1.5	1.6	1.8
7		1.2	1.3	1.3	1.4
6	3	1.0	1.0	1.0	1.1
5		0.79	0.79	0.81	0.97
4	2	0.60	0.60	0.62	0.66
3		0.42	0.43	0.43	0.46
2	1	0.27	0.27	0.27	0.29
1		<0.2	<0.2	<0.2	<0.2

Appendix 16: List of South Australian fields from which soil was sampled, including the property manager(s), field location, field description, soil type, GPS coordinates, year(s) canola was sown and quantity of *L. maculans* DNA in each sampling year, as estimated by the Root Disease Testing Service, SARDI. Field 21 is missing as it was selected but never sampled.

Field number	Property Manager(s)	Property location	Field description	Soil type	GPS coordinates	Canola sown	Blackleg DNA (pg/g soil)		
							2000	2001	2002
1	J & A Rohde	Tarlee	R2	RBE	S 34°18.523' E 138°45.875'	1995	0	2	0
2	J & A Rohde	Tarlee	Ke2	RBE	S 34°10.398' E 138°48.484'	2001	0	0	114
3	J & A Rohde	Tarlee	T3	RBE	S 34°18.523' E 138°45.875'	1993	12	9	6
4	R Agnew	Pt. Vincent	front of house	RBE over limestone	S 34°46.516' E 137°48.766'	N	1	2	0
5	R Agnew	Pt. Vincent	west of house	RBE over limestone	S 34°46.516' E 137°48.766'	N	10	0	6
6	R Agnew	Pt. Vincent	north of house	RBE over limestone	S 34°46.145' E 137°49.468'	N	28	0	6
7	M Weich	Kybunga	south of house	RBE	S 33°53.582' E 138°28.939'	1998	471	0	0
8	M Weich	Kybunga	over road	RBE	S 33°53.582' E 138°28.939'	N	0	0	6
9	M Weich	Kybunga	north of house	RBE	S 33°57.592' E 137°52.976'	N	4	6	6
10	N Crawford	Sandergrove	over railway	RBE	S 35°19.992' E 138°51.941'	1999	630	257	0
11	N Crawford	Sandergrove	south of Gale's	RBE	S 35°19.082' E 138°52.967'	2000	4	86	0
12	N Crawford	Sandergrove	opposite Charlick	RBE	S 35°19.082' E 138°52.967'	N	3	0	0
13	J Ayles	Willamulka	4	Sandy over heavier clay	S 33°57.594' E 137°52.979'	1999	471	18	0
14	J Ayles	Willamulka	2	Sandy over heavier clay	S 33°54.570' E 137°54.954'	2000	22	421	6
15	J Ayles	Willamulka	1	Sandy over heavier clay	S 33°54.476' E 137°54.416'	2001	0	0	858
16	J Ayles	Willamulka	3	Sandy over heavier clay	S 33°54.999' E 137°53.126'	N	0	0	0
17	J Ayles	Willamulka	5	Sandy over heavier clay	S 33°56.084' E 137°53.382'	N	0	0	0
18	L Gale	Sandergrove	railway	RBE	S 35°18.214' E 138°53.125'	1999	90	2	24
19	Lynch Farm Monitoring	Lock	shed paddock	Sandy	n/a	1998	0	0	ns
20	Lynch Farm Monitoring	Lock	strip	Sandy	n/a	2000	10	327	ns
22	Lynch Farm Monitoring	Lock	road	Sandy	n/a	N	0	0	ns
23	R Agnew	Pt. Vincent	front east house	RBE over limestone	S 34°46.775' E 137°48.876'	1998	9	8	6
24	J & A Rohde	Tarlee	Ki1	RBE	S 34°17.842' E138°46.075'	1996 & 2000	2	1067	90
25	J & A Rohde	Tarlee	T9	RBE	S 34°18.523' E 138°45.875'	1999	2215	2610	12

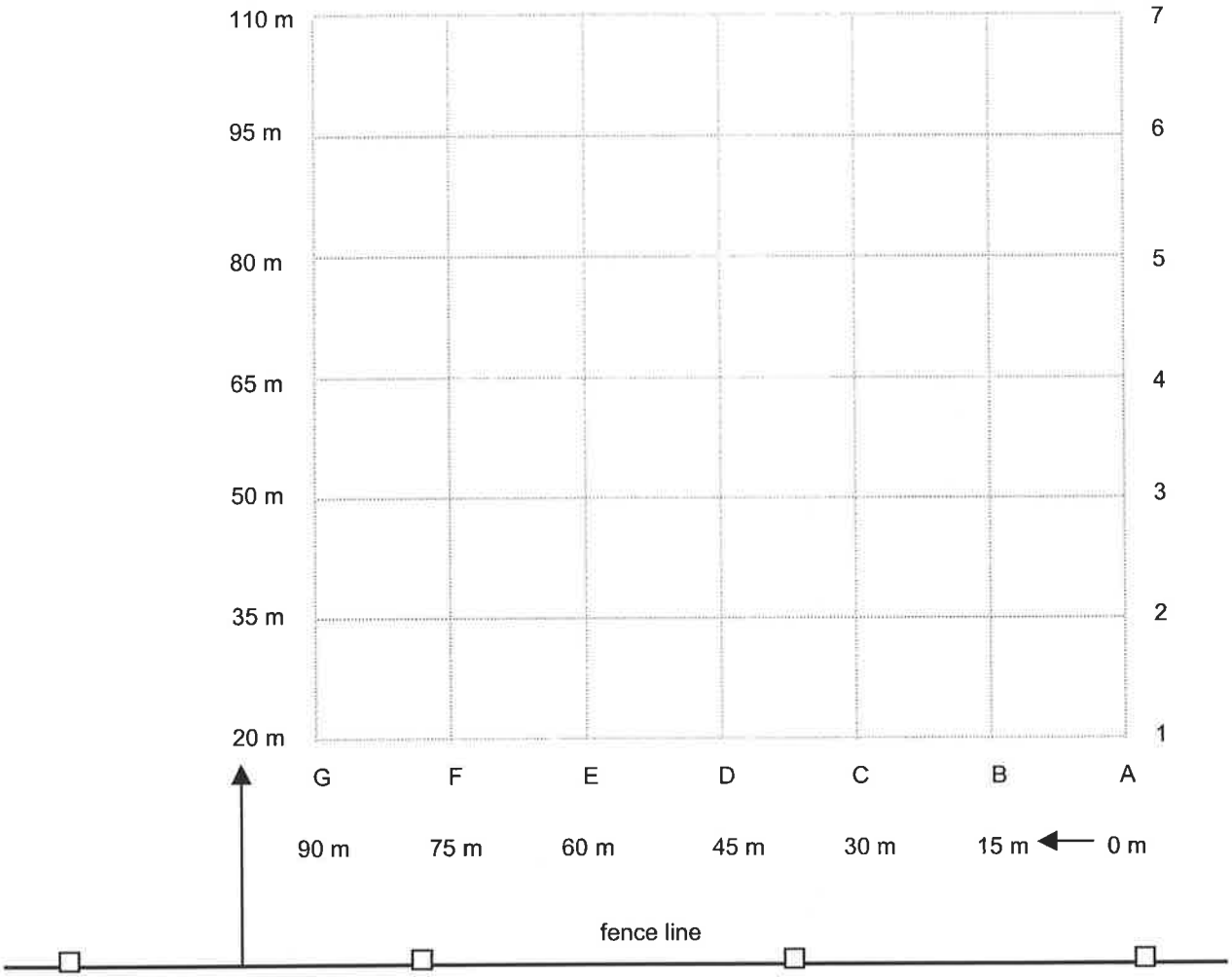
(continued)

Appendix 16: continued

Field number	Property Manager(s)	Property location	Field description	Soil type	GPS coordinates	Canola sown	Blackleg DNA (pg/g soil)		
							2000	2001	2002
26	J & A Rohde	Tarlee	Co1	RBE	S 34°18.795' E 138°45.039'	1998	14	0	0
27	J & A Rohde	Tarlee	W1	RBE	S 34°21.138' E 138°46.419'	1996 & 2001	8	6	534
28	J Ayles	Willamulka	Middle	Loam	n/a	N	ns	9	ns
29	M Wiech	Kybunga	DD5	Clay	n/a	N	ns	2	ns
30	M Wiech	Kybunga	DD120	Clay loam	n/a	2000	ns	215	ns
31	M Miller	Saddleworth	M3	Clay loam	n/a	N	ns	0	ns
32	M Miller	Saddleworth	K2	Clay loam	n/a	N	ns	6	ns
33	M Hill	Tarlee	Top river	Clay loam	n/a	N	ns	2	ns
34	A Laubsch	Kapunda	Vincint	Dark brown crac20 clay	n/a	N	ns	0	ns
35	A Laubsch	Kapunda	Black	Dark brown crac20 clay	n/a	1994 & 1999	ns	818	ns
36	N & S Lines	Wasleys	Top	Loam over heavy clay	n/a	N	ns	0	ns
37	N & S Lines	Wasleys	River	RBE over loam	n/a	N	ns	8	ns
38	N & S Lines	Wasleys	Banks	RBE over loam	n/a	N	ns	0	ns
39	N & S Lines	Wasleys	Gas	Loam over rubble	n/a	N	ns	1	ns
40	N & S Lines	Wasleys	Stable	Loam over rubble	n/a	N	ns	14	ns
41	D Crawford	Strathalbyn	Sandergrove	Sandy over loam	n/a	N	ns	15	ns
42	N Crawford	Strathalbyn	4 springs 1	Sandy over loam	n/a	N	ns	0	ns
43	N Crawford	Strathalbyn	4 springs 2	Sandy over loam	n/a	N	ns	7	ns
44	J & A Rohde	Tarlee	Mu1	RBE	S 34°19.187' E 138°45.000'	2000	ns	607	ns
45	J & A Rohde	Tarlee	R9	RBE	S 34°18.407' E 138°45.097'	1999	607	268	ns
46	J & A Rohde	Tarlee	T2	RBE	S 34°18.523' E 138°45.875'	1999	ns	34	ns
47	J & A Rohde	Tarlee	Ho1	RBE	S 34°20.261' E 138°46.046'	1998	ns	35	ns
48	J & A Rohde	Tarlee	Ko1	RBE	S 34°20.259' E 138°46.035'	1998	ns	47	ns
49	J & A Rohde	Tarlee	A2	RBE	S 34°19.801' E 138°45.074'	1997	ns	34	ns

RBE - red brown earth
n/a - not available
N - not sown to canola in at least 10 years
nc - fields in which canola has never been grown
ns - field not sampled in that year

Appendix 17: Grid sampling pattern used in the determination of sampling intensity necessary for collecting representative soil samples from a field.



Samples taken from the gridline intersections

Appendix 18: List of fields sampled in April 2001 on a property near Tarlee in South Australia, the year which canola was last grown, years since canola was grown, soil depth and the quantity of *L. maculans* DNA (pg/g soil) as estimated by the Root Disease Testing Service, SARDI.

Field name	Year canola last grown	Years since canola grown	Soil depth	*L. maculans* DNA (pg/g soil)
A2	1997	4	0-5cm	94
			5-10cm	0
			10-15cm	0
Co1	1998	3	0-5cm	22
			5-10cm	6
			10-15cm	0
Ho1	1998	3	0-5cm	1
			5-10cm	3
			10-15cm	81
Ko1	1998	3	0-5cm	92
			5-10cm	34
			10-15cm	0
R9	1999	2	0-5cm	703
			5-10cm	76
			10-15cm	5
T2	1999	2	0-5cm	18
			5-10cm	9
			10-15cm	54
Ki1	2000	1	0-5cm	148
			5-10cm	58
			10-15cm	0
Mu1	2000	1	0-5cm	1172
			5-10cm	69
			10-15cm	11
Ke2*	2001	1	0-5cm	791
			5-10cm	95
			10-15cm	35

*Field Ke2 was sampled in April 2002.

Appendix 19: List of fields sampled in April 2002 on a property near Tarlee in South Australia, the year which canola was last grown, years since canola was grown, OM fraction and the quantity of *L. maculans* DNA (pg/g soil) as estimated by the Root Disease Testing Service, SARDI.

Field name	Year canola last grown	Years since canola grown	OM fraction	*L. maculans* DNA (pg/g soil)
Ho1	1998	4	>2mm	1
			1.4-2mm	9
			0.5-1.4mm	23
			0.25-0.5mm	7
Co1	1998	4	>2mm	1
			1.4-2mm	-1
			0.5-1.4mm	2
			0.25-0.5mm	5
R9	1999	3	>2mm	16
			1.4-2mm	5
			0.5-1.4mm	5
			0.25-0.5mm	9
T2	1999	3	>2mm	0
			1.4-2mm	7
			0.5-1.4mm	115
			0.25-0.5mm	61
Mu1	2000	2	>2mm	35
			1.4-2mm	6
			0.5-1.4mm	19
			0.25-0.5mm	27
Ki1	2000	2	>2mm	310
			1.4-2mm	48
			0.5-1.4mm	69
			0.25-0.5mm	45
Ke2	2001	1	>2mm	11195
			1.4-2mm	3395
			0.5-1.4mm	1864
			0.25-0.5mm	577
W1	2001	1	>2mm	9866
			1.4-2mm	5176
			0.5-1.4mm	3841
			0.25-0.5mm	1508

REFERENCES

Agrios, G.N. (1997). *Plant Pathology.* Fourth edition. Academic Press, San Diego, California.

Angelini, R., Bragaloni, M., Federico, R., Infantino, A. and Porta-Puglia, A. (1993). Involvement of polyamines, diamine oxidase and peroxidase in resistance of chickpea to *Ascochyta rabiei*. *Journal of Plant Physiology* **142**: 704-709.

Angra-Sharma, R. and Sharma, D.K. (1994). Biochemical and histological studies on susceptible and resistant maize leaves infected by *Helminthosporium maydis*. *Plant Pathology* **43**: 972-978.

Annamali, P., Ishii, H., Lalithakumari, D. and Revathi, R. (1995). Polymerase chain reaction and its application in fungal disease diagnosis. *Journal of Plant Diseases and Protection* **102**: 91-104.

Annis, S.L. and Goodwin, P.H. (1997a). Inhibition of polygalacturonase activity produced by *Leptosphaeria maculans* with stem extracts of canola, and the relationship of inhibition to resistance. *Journal of Phytopathology* **145**: 217-223.

Annis, S.L. and Goodwin, P.H. (1997b). Inhibition of polygalacturonase activity produced by *Leptosphaeria maculans* by leaf extracts of canola and its relationship to calcium. *Canadian Journal of Plant Pathology* **19**: 1-7.

Anonymous (1994a). *Canola News*. Canola Association of Australia. Issue **43** Feb 1994.

Anonymous (1994b). Statistix® - Version 4.1 User's Manual. Analytical Software, Tallahasse, Florida, USA.

Anonymous (1996). Saskatchewan Agriculture and Food, Farmfacts: Blackleg - A Disease of Canola. [www.gov.sk.ca]

Anonymous (1999). *Australian Oilseed News*. Australian Oilseeds Federation. May 1999.

Anonymous (2002a). *Canola News*. Canola Association of Australia. Issue **75** July 2002.

Anonymous (2002b). *Field Crops Disease Spraying Chart 2002.* Primary Industries and Resources SA. Agdex 110/640.

Ansan-Melayah, D., Rouxel, T., Bertrandy, J., Letarnec, B., Mendes-Pereira, E. and Balesdent, M.H. (1997). Field efficiency of *Brassica napus* specific resistance correlates with *Leptosphaeria maculans* population structure. *European Journal of Plant Pathology* **103**: 835-841.

Badawy, H.M.A., Hoppe, H.H. and Koch, E. (1991). Differential reactions between the genus *Brassica* and aggressive single spore isolates of *Leptosphaeria maculans*. *Journal of Phytopathology* **131**: 109-119.

Bain, R.A., Millard, P. and Perombelon, M.C.M. (1996). The resistance of potato plants to *Erwinia carotovora* subsp. *atroseptica* in relation to their calcium and magnesium content. *Potato Research* **39**:185-193.

Baird, R.E., Phillips, D.V., Mullinix, B.G. and Alt, P.J. (1999). Relative longevity of *Leptosphaeria maculans* and associated mycobiota on canola debris. *Phytoprotection* **80**: 1-11.

Balali, G.R., Whisson, E.L., Scott, E.S. and Neate, S.M. (1996). DNA fingerprinting probe specific to isolates of *Rhizoctonia solani* AG-3. *Mycological Research* **100**: 467-470.

Balesdent, M.H., Gall, C., Robin, P. and Rouxel, T. (1992). Intraspecific variation in soluble mycelial protein and esterase patterns of *Leptosphaeria maculans* French isolates. *Mycological Research* **96**: 677-684.

Balesdent, M.H., Jedryczka, M., Jain, L., Mendes-Pereira, E., Bertrandy, J. and Rouxel, T. (1998). Conidia as a substrate for internal transcribed spacer-based PCR identification of members of the *Leptosphaeria maculans* species complex. *Phytopathology* **88**: 1210-1217.

Ballinger, D.J. and Salisbury, P.A. (1996). Seedling and adult plant evaluation of race variability in *Leptosphaeria maculans* on brassica species in Australia. *Australian Journal of Experimental Agriculture* **36**: 485-488.

Ballinger, D.J., Salisbury, P.A., Dennis, J.I., Kollmorgen, J.F. and Potter, T.D. (1988). Evaluation of rates of flutriafol for control of blackleg of rapeseed. *Australian Journal of Experimental Agriculture* **28**: 517-519.

Barbetti, M.J. (1975). Effects of temperature on development and progression in rape crown canker caused by *Leptosphaeria maculans*. *Australian Journal of Experimental Agriculture and Animal Husbandry* **15**: 705-708.

Barbetti, M.J. (1976). The role of pycnidiospores of *Leptosphaeria maculans* in the spread of blackleg disease in rape. *Australian Journal of Experimental Agriculture and Animal Husbandry* **16**: 911-914.

Barbetti, M.J. (1978). Infection of oilseed rape and cruciferous weeds with *Leptosphaeria maculans* isolates from oilseed rape and wild radish. *Australian Plant Pathology Society Newsletter* **7**: 3-5.

Barbetti, M.J., Carmody, P., Khangura, R.K., Sweetingham, M. and Walton, G. (2000). *Managing blackleg in 2000.* Perth, Agriculture Western Australia, Bulletin no. 4400.

Barbetti, M.J. and Khangura, R.K. (1997). Developments for better management of blackleg disease in Western Australia. *11th Australian Research Assembly on Brassicas, 1997, Perth, Western Australia* pp 11-14.

Barbetti, M.J. and Khangura, R.K. (1999). Managing blackleg in the disease-prone environment of Western Australia. *10th International Rapeseed Congress, 1999, Canberra, Australia* p 100.

Barbetti, M.J. and Khangura, R.K. (2000). *Fungal Diseases of Canola in Western Australia.* Perth, Agriculture Western Australia, Bulletin no. 4406.

Barrins, J.M., Purwantara, A., Ades, P.K., Salisbury, P.A. and Howlett, B.J. (2002). Genetic diversity of isolates of *Leptosphaeria maculans* from a canola (*Brassica napus*) paddock in Australia. *Australasian Plant Pathology* **31**: 129-135.

Benhamou, N. and Chet, I. (1996). Parasitism of sclerotia of *Sclerotium rolfsii* by *Trichoderma harzianum*: ultrastructural and cytochemical aspects of the interaction. *Phytopathology* **86**: 405-416.

Benhamou, N. and Chet, I. (1997). Cellular and molecular mechanisms involved in the interaction between *Trichoderma harzianum* and *Pythium ultimum*. *Applied and Environmental Microbiology*. **63**: 2095-2099.

Biddulph, J.E., Fitt, B.D.L., Leech, P.K., Welham, S.J. and Gladders, P. (1999). Effects of temperature and wetness duration on infection of oilseed rape leaves by ascospores of *Leptosphaeria maculans* (stem canker). *European Journal of Plant Pathology* **105**: 769-781.

Blenis, P.V., Chow, P.S. and Stringam, G.R. (1999). Effects of burial, stem portion and cultivar on the decomposition of canola straw. *Canadian Journal of Plant Science* **79**: 97-100.

Bokor, A., Barbetti, M.J., Brown, A.G.P., MacNish, G.C. and Wood, P.M. (1975). Blackleg of rapeseed. *Journal of Agriculture, Western Australia* **16**: 7-10.

Booth, C. (1971). *Methods in Microbiology*. Volume 4. Academic Press, London, United Kingdom.

Bretag, T.W. (1991). Epidemiology and control of ascochyta blight of field peas. Ph.D Thesis, La Trobe University, Victoria, Australia. p 91.

Brill, L.M., McClary, R.D. and Sinclair, J.B. (1994). Analysis of two ELISA formats and antigen preparations using polyclonal antibodies against *Phomopsis longicolla*. *Phytopathology* **84**: 173-179.

Brown, A.G.P., Barbetti, M.J. and Wood, P.McR. (1976). Effect of benomyl on 'blackleg' disease of rape in Western Australia. *Australian Journal of Experimental Agriculture and Animal Husbandry* **16**: 276-279.

Brun, H., Levivier, S, Somda, I., Ruer, D., Renard, M. and Chèvre, A.M. (2000). A field method for evaluating the potential durability of new resistance sources: Application to the *Leptosphaeria maculans-Brassica napus* pathosystem. *Phytopathology* **90**: 961-966.

Buzza, G.C. (1995). Plant Breeding. In: *Brassica Oilseeds* – Production and Utilization. eds: Kimber, D.S. and McGregor, D.I. CAB International, Wallingford, United Kingdom. Pp. 153-175.

Buzza, G. and Easton, A (2002). A new source of blackleg resistance from *Brassica sylvestris*. Groupe Consultatif International de Recherche sur le Colza. Bulletin B18, Technical meeting, Poznan, Poland, June 2002.

Calderon, C., Ward, E., Freeman, J., Foster, S.J. and McCartney, H.A. (2002). Detection of airborne inoculum of *Leptosphaeria maculans* and *Pyrenopeziza brassicae* in oilseed rape crops by polymerase chain reaction (PCR) assays. *Plant Pathology* **51**: 303-310.

Cargeeg, L.A. and Thurling, N. (1980). Contribution of host-pathogen interactions to the expression of the blackleg disease of spring rape caused by *Leptosphaeria maculans*. *Euphytica* **29**: 465-476.

Chen C.Y. and Howlett B.J. (1996). Rapid necrosis of guard cells is associated with the arrest of fungal growth in leaves of Indian mustard (*Brassica juncea*) inoculated with avirulent isolates of *Leptosphaeria maculans*. *Physiological and Molecular Plant Pathology* **48**: 73-81.

Chen, C.Y. and Seguin-Swartz, G. (1999). Reaction of wild crucifers to *Leptosphaeria maculans*, the causal agent of blackleg of crucifers. *Canadian Journal of Plant Pathology* **21**: 361-367.

Chèvre, A.M., Barret, P., Eber, F., Dupuy, P., Brun, H., Tanguy, X. and Renard, M. (1997). Selection of stable *Brassica napus-B. juncea* recombinant lines resistant to blackleg (*Leptosphaeria maculans*). 1. Identification of molecular markers, chromosomal and genomic origin of the introgression. *Theoretical and Applied Genetics* **95**: 1104-1111.

Chèvre, A.M., Eber, F., This, P., Barret, P., Tanguy, X., Brun, H., Delseny, M. and Renard, M. (1996). Characterization of *Brassica nigra* chromosomes and of blackleg resistance in *B. napus-B. nigra* addition lines. *Plant Breeding* **115**: 113-118.

Colton, B. and Potter, T. (1999). History. In: *Canola in Australia – The First 30 Years*. Eds. Salisbury, P.A., Potter, T.D., McDonald, G. and Green, A.G. Canola Association of Australia. pp 1-4.

Crouch, J.H., Lewis, B.G. and Mithen, R.F. (1994). The effect of A genome substitution on the resistance of *Brassica napus* to infection by *Leptosphaeria maculans*. *Plant Breeding* **112**: 265-278.

Curtis, H. and Barnes, N.S. (1989). *Biology*. 5th edition. Worth Publishers Inc., New York, United States of America.

Dai, G.H., Andary, C., Mondolot-Cosson, L. and Boubals, D. (1995). Histochemical studies on the interaction between three species of grapevine, *Vitis vinifera, V. rupestris* and *V. rotundifolia* and the downy mildew fungus, *Plasmopara viticola*. *Physiological and Molecular Plant Pathology* **46**: 177-188.

D'Arcy, C.J., Hewings, A.D. and Eastman, C.E. (1992). Reliable detection of barley yellow dwarf viruses in field samples by monoclonal antibodies. *Plant Disease* **76**: 273-276.

Davidson, J.A., Ophel-Keller, K., Hartley, D., Krysinska-Kaczmarek, M., Curran, J. and Ramsey, M.D. (2001). Monitoring blackspot pathogens in pea cropping soils. *Second Australasian Soilborne Diseases Symposium, March 2001*. pp 33-34.

Davidson, J., Ramsey, M. and Sosnowski, M. (1999). The relationship between soil-borne pathogens and disease in field peas (*Pisum sativum*). *First Australasian Soilborne Disease Symposium, Feb. 1999*. pp 187-188.

De March, G., Séguin-Swartz, G. and Petrie, G.A. (1986). Virulence and culture filtrate phytotoxicity in *Leptosphaeria maculans*: Perspectives for in vitro selection. *Canadian Journal of Plant Pathology* **8**: 422-428.

Dhingra, O.D. and Sinclair, J.B. (1995). Basic Plant Pathology Methods (2nd Edition). CRC Press, Boca Raton, Florida. p 393.

Dixelius, C. and Wahlberg, S. (1999). Resistance to *Leptosphaeria maculans* is conserved in a specific region of the *Brassica* B genome. *Theoretical and Applied Genetics* **99**: 368-372.

Downey, R.K., Pawlowski, S.H. and Mc Ansh, J. (1970). Rapeseed – Canada's "cinderella" crop. Ed 2. Rapeseed Association of Canada, Pub. 8.

Dushnicky, L.G., Ballance, G.M., Sumner, M.J. and MacGregor, A.W. (1998). The role of lignification as a resistance mechanism in wheat to a toxin-producing isolate *of Pyrenophora tritici-repentis. Canadian Journal of Plant Pathology* **20**: 35-47.

Edel, V. (1998). Polymerase chain reaction in mycology: an overview. In: *Applications of PCR in Mycology.* Eds. Bridge, P.D., Arora, D.K., Reddy, C.A. and Elander, R.P. CAB International, Oxon, UK.

Edwards, S.G., Fitter, A.H. and Young, J.P.W. (1997). Quantification of an arbuscular mycorrhizal fungus, *Glomus mosseae*, within plant roots by competitive polymerase chain reaction. *Mycological Research* **101**: 1440-1444.

Evans, K.J., Whisson, D.L. and Scott, E.S. (1996). An experimental system for characterising isolates of *Uncinula necator. Mycological Research* **100**: 675-680.

FAO, 2002. From FAOSTAT on-line statistical database. *Food and Agriculture Organisation of the United Nations*. [http://apps.fao.org/default.htm]

Faulkner, G. and Kimmins, W.C. (1975). Staining reactions of the tissue bordering lesions induced by wounding, tobacco mosaic virus, and tobacco necrosis virus in bean. *Phytopathology* **65**: 1396-1400.

Fieldsend, J. and Milford, G.F.J. (1994). Changes in glucosinolates during crop development in single- and double-low genotypes of winter oilseed rape (*Brassica napus*): II. Profiles and tissue-water concentrations in vegetative tissues and developing pods. *Annals of Applied Biology* **124**: 543-555.

Finch, R.P. (1994). An introduction to molecular biology. In: *Molecular Biology in Crop Protection.* Eds. Marshall, G. and Walters, D. Chapman and Hall, London, UK.

Friend, J. (1976). Lignification in infected tissue. In: *Biochemical Aspects of Plant-Parasite Relationships*. Eds. Friend, J. and Threlfall, D.R. Academic Press, New York pp 291-303.

Fox, R.T.V (1997). The present and future use of technology to detect plant pathogens to guide disease control in sustainable farming systems. *Agriculture, Ecosystems and Environment* **64**: 125-32.

Gabrielson, R.L. (1983). Blackleg disease of crucifers caused by *Leptosphaeria maculans* (*Phoma lingam*) and its control. *Seed Science Technology* **11**: 749-780.

Giamoustaris, A. and Mithen, R. (1997). Glucosinolates and disease resistance in oilseed rape (*Brassica napus* ssp. *oleifera*). *Plant Pathology* **46**: 271-275.

Gladders, P. and Musa, T.M. (1980). Observations on the epidemiology of *Leptosphaeria maculans* stem canker in winter oilseed rape. *Plant Pathology* **29**: 28-37.

Goodwin, P.H. and Annis, S.L. (1991). Rapid identification of genetic variation and pathotype of *Leptosphaeria maculans* by random amplified polymorphic DNA assay. *Applied and Environmental Microbiology* **57**: 2482-2486.

Gugel, R.K. and Petrie, G.A. (1992). History, occurrence, impact and control of blackleg of rapeseed. *Canadian Journal of Plant Pathology* **14**: 36-45.

Gugel, R.K., Séguin-Swartz, G. and Petrie, G.A. (1990). Pathogenicity of three isolates of *Leptosphaeria maculans* on *Brassica* species and other crucifers. *Canadian Journal of Plant Pathology* **12**: 75-82.

Hall, R. (1992). Epidemiology of blackleg of oilseed rape. *Canadian Journal of Plant Pathology* **4**: 46-55.

Hall, R., Peters, R.D. and Assabgui, R.A. (1993). Occurrence and impact of blackleg of oilseed rape in Ontario. *Canadian Journal of Plant Pathology* **15**: 305-313.

Hall, R., Chigogora, J.L. and Phillips, L.G. (1996). Role of seedborne inoculum of *Leptosphaeria maculans* in development of blackleg on oilseed rape. *Canadian Journal of Plant Pathology* **18**: 35-42.

Hammond, K.E. and Lewis, B.G. (1986a). The timing and sequence of events leading to stem canker disease in populations of *Brassica napus* var. *oleifera* in the field. *Plant Pathology* **35**: 551-564.

Hammond, K.E. and Lewis, B.G. (1986b). Ultrastructural studies of the limitation of lesions caused by *Leptosphaeria maculans* in stems of *Brassica napus* var. *oleifera. Physiological and Molecular Plant Pathology* **28**: 251-265.

Hammond, K.E. and Lewis, B.G. (1987a). The establishment of systemic infection in leaves of oilseed rape by *Leptosphaeria maculans. Plant Pathology* **36**: 135-147.

Hammond, K.E. and Lewis, B.G. (1987b). Variation in stem infections caused by aggressive and non-aggressive isolates of *Leptosphaeria maculans* on *Brassica napus* var. *oleifera. Plant Pathology* **36**: 53-65.

Hammond, K.E., Lewis, B.G. and Musa, T.M. (1985). A systemic pathway in the infection of oilseed rape plants by *Leptosphaeria maculans. Plant Pathology* **34**: 557-565.

Hanacziwskyj, P. and Drysdale, R.B. (1984). Variation in pathogenicity of *Leptosphaeria maculans* to oilseed rape and other brassicas. *Aspects of Applied Biology* **6:** 343-353.

Hare, R.C. (1966). Physiology of resistance to fungal diseases in plants. *The Botanical Review* **32**: 95-137.

Harper, F.R. and Berkenkamp, B. (1975). Revised growth-stage key for *Brassica campestris* and *B. napus. Canadian Journal of Plant Science* **55**: 657-658.

Henderson, M.P. (1918). The black-leg disease of cabbage caused by *Phoma lingam* (Tode) Desmaz. *Phytopathology* **8**: 379-431.

Henderson, M. (2000). *Biological Scanning Electron Microscopy.* Centre for Electron Microscopy and Microstructure Analysis, The University of Adelaide, Australia.

Herdina and Roget, D.K. (2000). Prediction of take-all disease risk in field soils using a rapid and quantitative DNA soil assay. *Plant and Soil* **227**: 87-98.

Herdina, Yang, H.A. and Ophel-Keller, K. (1997). Correlation of take-all disease severity and inoculum level of *Gaeumannomyces graminis* var. *tritici* using a slot-blot hybridization assay. *Mycological Research* **101**: 1311-1317.

Hershman, D.E. and Perkins, D.M. (1995). Etiology of canola blackleg in Kentucky and seasonal discharge patterns of *Leptosphaeria maculans* ascospores from infested canola stubble. *Plant Disease* **79**: 1225-1229.

Hocking, A.D. and Pitt, J.L. (1980). Dichloran-Glycerol medium for enumeration of xerophilic fungi from low-moisture foods. *Applied and Environmental Microbiology.* **39**: 488-492.

Holliday, P. (1989). *A Dictionary of Plant Pathology.* Cambridge University Press, Cambridge, UK.

Howlett, B.J., Idnurm, A. and Pedras, M.S.C. (2001). *Leptosphaeria maculans,* the causal agent of blackleg disease of brassicas [Review]. *Fungal Genetics and Biology* **33**: 1-14.

Huang, Y.J., Toscano-Underwood, C., Fitt, B.D.L., Todd, A.D., West, J.S., Koopmann, B. and Balesdent, M.H. (2001). Effects of temperature on germination and hyphal growth from ascospores of A-group and B-group *Leptosphaeria maculans* (phoma stem canker of oilseed rape). *Annals of Applied Biology* **139**: 193-207.

Ihaka, R. and Gentleman, R. (1996). R: A Language for Data Analysis and Graphics. *Journal of Computational and Graphical Statistics* **5**: 299-314.

Jabaji-Hare, S., Chamberland, H. and Charest, P.M. (1999). Cell wall alterations in hypocotyls of bean seedlings protected from Rhizoctonia stem canker by a binucleate *Rhizoctonia* isolate. *Mycological Research* **103**: 1035-1043.

Jedryczka, M., Rouxel, T. and Balesdent, M.H. (1999). Rep-PCR based genomic fingerprinting of isolates of *Leptosphaeria maculans* from Poland. *European Journal of Plant Pathology* **105:** 813-823.

Jensen, W.A. (1962). *Botanical Histochemistry.* Freeman, San Francisco, California, USA.

Johnson, R.D. and Lewis, B.G. (1994). Variation in host range, systemic infection and epidemiology of *Leptosphaeria maculans. Plant Pathology* **43**: 269-277.

Judelson, H.S. and Messenger-Routh, B. (1996). Quantification of *Phytophthora cinnamomi* in avocado roots using a species-specific DNA probe. *Phytopathology* **86**: 763-768.

Khan, M.S.A., Ramsey, M.D., Corbière, R., Infantino, A., Porta-Puglia, A., Bouznad, Z. and Scott, E.S. (1999). Ascochyta blight of chickpea in Australia: identification, pathogenicity and mating type. *Plant Pathology* **48**: 230-234.

Khangura, R.K. and Barbetti, M.J. (2002). Efficacy of Impact to manage blackleg (*Leptosphaeria maculans*) in canola. *Australian Journal of Agricultural Research* **53**: 311-321.

Khangura, R.K. and Barbetti, M.J. (2001). Prevalence of blackleg (*Leptosphaeria maculans*) on canola (*Brassica napus*) in Western Australia. *Australian Journal of Experimental Agriculture* **41**: 71-80.

Khangura, R.K., Barbetti, M.J. and Sweetingham, M.W. (1999). Characterization and pathogenicity of *Rhizoctonia* species on canola. *Plant Disease* **83**: 714-721.

Khangura, R.K., Barbetti, M.J. and Walton, G. (2002). WA blackleg resistance ratings on canola varieties for 2002. Department of Agriculture Western Australia, Farmnote 02/2002.

Kharbanda, P.D. and Ostashewski, M.J. (1997). Influence of burying blackleg infected canola stubble on pseudothecia formation in *Leptosphaeria maculans*. *Canadian Journal of Plant Pathology* **19**: 111.

Kharbanda, P.D. and Tewari, J.P. (1996). Integrated management of canola diseases using cultural methods. *Canadian Journal of Plant Pathology* **18**: 168-175.

Kharbanda, P.D., Yang, J., Beatty, P., Jensen, S. and Tewari J.P. (1999). Biocontrol of *Leptosphaeria maculans* and other pathogens of canola with *Paenibacillus polymyxa* PKB1. *10th International Rapeseed Congress, 1999, Canberra, Australia* p 228.

King, A. D., Hocking, A.D. and Pitt, J.I. (1979). Dichloran-Rose Bengal medium for enumeration and isolation of molds from foods. *Applied and Environmental Microbiology* **37**: 959-964.

Klassen, G.R., Balzerzak, M. and de Cock, W.A.M. (1996). 5S ribosomal RNA gene spacers as species-specific probes for eight species of *Pythium*. *Phytopathology* **86**: 581-587.

Koch, E., Badawy, H.M.A. and Hoppe, H.H. (1989). Differences between aggressive and nonaggressive single spore lines of *Leptosphaeria maculans* in cultural characteristics and phytotoxin production. *Journal of Phytopathology* **124**: 52-62.

Koch, E., Song, K., Osborn, T.C. and Williams, P.H. (1991). Relationship between pathogenicity and phylogeny based on restriction fragment length polymorphism in *Leptosphaeria maculans*. *Molecular Plant Microbe Interactions* **4**: 341-349.

Kutcher, H.R., van den Berg, C.G.J. and Rimmer, S.R. (1993). Variation in pathogenicity of *Leptosphaeria maculans* on *Brassica* spp. based on cotyledon and stem reactions. *Canadian Journal of Plant Pathology* **15**: 253-258.

Kuusk, A.K., Happstadius, I., Zhou, L., Steventon, L.A., Giese, H. and Dixelius, C. (2002). Presence of *Leptosphaeria maculans* Group A and Group B isolates in Sweden. *Journal of Phytopathology* **150**: 349-356.

Lamont, J. (1990). Canola industry background. Canola Association of Australia Inc., New South Wales Government Printing Service pp 1-14.

Lewis, D. (1998). Crop and Pasture Report. Primary Industries and Resources of South Australia. June, 1998.

MacNish, G.C. (1979). Survival of *Leptosphaeria maculans* in rapeseed root tissue. *Australasian Plant Pathology* **8**: 23-24.

Mahuku, G.S., Goodwin, P.H., Hall, R. and Hsiang T. (1997). Variability in the highly virulent type of *Leptosphaeria maculans* within and between oilseed rape fields. *Canadian Journal of Botany* **75**: 1485-1492.

Mahuku, G.S., Hall, R. and Goodwin, P.H. (1996). Distribution of *Leptosphaeria maculans* in two fields in southern Ontario as determined by the polymerase chain reaction. *European Journal of Plant Pathology* **102**: 569-576.

Manici, L.M., Lazzeri, L. and Palmieri, S. (1997). *In vitro* fungitoxic activity of some glucosinolates and their enzyme-derived products toward plant pathogenic fungi. *Journal of Agricultural and Food Chemistry* **45**: 2768-2773.

Marcroft, S. (2001). New national canola blackleg rating system developed for 2001. *Cropping Systems Update 2001, Adelaide, South Australia, March 2001.* p. 37.

Marcroft, S.J., Purwantara, A., Salisbury, P.A., Potter, T.D., Wratten, N., Khangura, R., Barbetti, M.J. and Howlett, B.J. (2002). Reaction of a range of *Brassica* species under Australian conditions to the fungus, *Leptosphaeria maculans,* the causal agent of blackleg. *Australian Journal of Experimental Agriculture* **42**: 587-594.

Marcroft, S.J., Sprague, S.J., Salisbury, P.A. and Howlett, B.J. (2001). Blackleg epidemiology in Australia. *12th Australian Research Assembly on Brassicas, 2001, Geelong, Victoria* pp 78-81.

McGee, D.C. (1977). Blackleg (*Leptosphaeria maculans* (Desm. Ces. et de Not.)) of rapeseed in Victoria: sources of infection and relationships between inoculum, environmental factors and disease severity. *Australian Journal of Agricultural Research* **28**: 53-62.

McGee, D.C. and Emmett, R.W. (1977). Blackleg (*Leptosphaeria maculans*(Desm.) Ces. et de Not.) of rapeseed in Victoria: Crop losses and factors which affect disease severity. *Australian Journal of Agricultural Research* **28**: 47-51.

McGee, D.C. and Petrie, G.A. (1978). Variability of *Leptosphaeria maculans* in relation to blackleg of oilseed rape. *Phytopathology* **68**: 625-630.

McGee, D.C. and Petrie, G.A. (1979). Seasonal patterns of ascospore discharge by *Leptosphaeria maculans* in relation to blackleg of oilseed rape. *Phytopathology* **69**: 586-589.

Mengistu, A., Rimmer, S.R., Koch, E. and Williams, P.H. (1991). Pathogenicity grouping of isolates of *Leptosphaeria maculans* on *Brassica napus* cultivars and their disease reaction profiles in rapid-cycling brassicas. *Plant Disease* **75**: 1279-1282.

Michelmore, R.W. and Hulbert, S.H. (1987). Molecular markers for genetic analysis of phytopathogenic fungi. *Annual Review of Phytopathology* **25**: 383-404.

Miller, S.A. (1995). Plant disease diagnosis: biotechnological approaches. In: *Molecular Methods in Plant Pathology.* Eds. Singh, R.P. and Singh, U.S. CRC Press, Boca Raton, Florida, USA.

Miller, S.A. and Martin, R.R. (1988). Molecular diagnosis of plant disease. *Annual Review of Phytopathology* **26**: 409-432.

Misaghi, I.J. (1982). *Physiology and Biochemistry of Plant-Pathogen Interactions.* Plenum Press, New York, USA. pp 149-182.

Mithen, R.F. and Lewis, B.G. (1988). Resistance to *Leptosphaeria maculans* in hybrids of *Brassica oleracea* and *Brassica insularis*. *Journal of Phytopathology* **123**: 253-258.

Mithen, R.F., Lewis, B.G. and Fenwick, G.R. (1986). *In vitro* activity of glucosinolates and their products against *Leptosphaeria maculans*. *Transactions of the British mycological Society* **87**: 433-440.

Mithen, R.F., Lewis, B.G., Heaney R.K and Fenwick, G.R. (1987). Resistance of leaves of *Brassica* species to *Leptosphaeria maculans*. *Transactions of the British mycological Society* **88**: 525-531.

Morales, V.M., Seguin-Swartz, G. and Taylor, J.L. (1993). Chromosome size polymorphism in *Leptosphaeria maculans*. *Phytopathology* **83**: 503-509.

Mordue, J.E.M. (1974). *Thanatephorus cucumeris (Rhizoctonia solani).* CMI Descriptions of Pathogenic Fungi and Bacteria. No. 406. The Eastern Press Ltd, London, UK.

Moury, B., Cardin, L., Onesto, J-P., Candresse T. and Poupet, A. (2000). Enzyme-linked immunosorbent assay testing of shoots grown in vitro and the use of immunocapture-reverse transcription-polymerase chain reaction improve the detection of *Prunus ringspot virus* in rose. *Phytopathology* **90**: 522-528.

Mouzeyar, S., Tourvieille de Labrouhe, D. and Vear, F. (1993). Histopathological studies of resistance of sunflower (*Helianthus annuus* L.) to downy mildew (*Plasmopara halstedii*). *Journal of Phytopathology* **139**: 289-297.

Narayanasamy, P. (1997). *Plant Pathogen Detection and Disease Diagnosis.* Marcel Dekker Inc., New York, USA.

Nathaniels, N.Q.R. and Taylor, G.S. (1983). Latent infection of winter oilseed rape by *Leptosphaeria maculans*. *Plant Pathology* **32**: 23-31.

Nelson, P.E., Toussoun, T.A. and Marasas, W.F.O. (1983). *Fusarium* species: An illustrated manual for identification. The Pennsylvania State University Press, University Park, USA. p 8.

Newman, P.L. (1984). Differential host-parasite interactions between oilseed rape and *Leptosphaeria maculans,* the causal fungus of stem canker. *Plant Pathology* **33**: 205-210.

Newman, P.L. and Bailey, D.J. (1987). Screening for resistance to canker (*Leptosphaeria maculans*) in winter oilseed rape (*Brassica napus* ssp. *oleifera*). *Plant Pathology* **36**: 346-354.

Nuzum, C. and Kaldor, C.J. (1988). Assessment and control of latent infection of rape, *Brassica napus*, by *Leptosphaeria maculans*. *Australasian Plant Pathology* **17**: 74-78.

Ophel-Keller, K., Engel, B. and Heinrich, K. (1995). Specific detection of *Gaeumannomyces graminis* in soil using polymerase chain reaction. *Mycological Research* **41**: 554-562.

Ophel-Keller, K., McKay, A., Driver, F. and Curran, J. (1999). The cereal root disease testing service. *First Australasian Soilborne Disease Symposium,Gold Coast, Australia Feb. 1999*. pp 63-64.

Papavizas, G.C. (1985). *Trichoderma* and *Gliocladium*: biology, ecology, and potential for biocontrol. *Annual Review of Phytopathology* **23**: 23-54.

Pedras, M.S.C. and Biesenthal, J. (2000). HPLC analysis of cultures of *Phoma* spp.: Differentiation among groups and species through secondary metabolite profiles. *Canadian Journal of Microbiology* **46**: 685-691.

Pérès, A. Poisson, B., LeSourne, V. and Maisonneuve, C. (1999). *Leptosphaeria maculans*: effect of temperature, rainfall and humidity on the formation of pseudothecia. *10th International Rapeseed Congress, 1999, Canberra, Australia* p 133.

Petrie, G.A. (1978). Occurrence of a highly virulent strain of blackleg (*Leptosphaeria maculans*) on rape in Saskatchewan (1975-1977). *Canadian Plant Disease Survey* **58**: 21-25.

Petrie, G.A (1985). Sexual compatibility of isolates of the rapeseed blackleg fungus *Leptosphaeria maculans* from Canada, Australia and England. *Canadian Journal of Plant Pahology* **7**: 253-255.

Petrie, G.A. (1986). Consequences of survival of *Leptosphaeria maculans* (blackleg) in canola stubble residue through an entire crop rotation sequence. *Canadian Journal of Plant Pathology* **8**: 353 (Abstract).

Petrie, G.A. (1994). Effects of temperature and moisture on the number, size and septation of ascospores produced by *Leptosphaeria maculans* (blackleg) on rapeseed stubble. *Canadian Plant Disease Survey* **74**: 141-151.

Petrie, G.A. (1995a). Effects of chemicals on ascospore production by *Leptosphaeria maculans* on blackleg-infected canola stubble in Saskatchewan. *Canadian Plant Disease Survey* **75**: 45-50.

Petrie, G.A. (1995b). Long-term survival and sporulation of *Leptosphaeria maculans* (blackleg) on naturally-infected rapeseed/canola stubble in Saskatchewan. *Canadian Plant Disease Survey* **75**: 23-34.

Petrie, G.A. and Lewis, P.A. (1985). Sexual compatibility of isolates of the rapeseed blackleg fungus *Leptosphaeria maculans* from Canada, Australia and England. *Canadian Journal of Plant Pathology* **7**: 253-255.

Plummer, K.M., Dunse K. and Howlett, B.J. (1994). Non-aggressive strains of the blackleg fungus, *Leptosphaeria maculans*, are present in Australia and can be distinguished from aggressive strains by molecular analysis. *Australian Journal of Botany* **42**: 1-8.

Poisson, B. and Pérès, A. (1999). Study of rapeseed susceptibility to primary contamination of *Leptosphaeria maculans* in relation to plant vegetative stage. *10th International Rapeseed Congress, 1999, Canberra, Australia* p 135.

Politis, D.J. and Goodman, R.N. (1978). Localised cell wall appositions: Incompatibility response of tobacco leaf cells to *Pseudomonas pisi*. *Phytopathology* **68**: 309-316.

Potter, T. and Stanley, M. (2002). Canola variety sowing guide for 2002. Fact sheet. Primary Industries and Resources of South Australia & South Australian Research and Development Institute. Agdex 144/10, ISSN 1440-8775.

Punithalingam, E. and Holliday, P. (1972). *Leptosphaeria maculans.* CMI Descriptions of Pathogenic Fungi and Bacteria. No. 331. The Eastern Press Ltd, London, UK.

Purwantara, A., Barrins, J.M., Cozijnsen, A.J., Ades, P.K. and Howlett, B.J. (2000). Genetic diversity of isolates of the *Leptosphaeria maculans* species complex from Australia, Europe and North America using amplified fragment length polymorphism analysis. *Mycological Research* **104**: 772-781.

Putnam, M.L. (1995). Evaluation of selected methods of plant disease diagnosis. *Crop Protection* **14**: 517-525.

Randles, J.W., Hodgson, R.A.J. and Wefels, E. (1996). The rapid and sensitive detection of plant pathogens by molecular methods. *Australasian Plant Pathology* **25**: 71-85.

Rempel, C.B. and Hall, R. (1993). Dynamics of production of ascospores of *Leptosphaeria maculans* in autumn on stubble of the current year's crop of spring rapeseed. *Canadian Journal of Plant Pathology* **15**: 182-184.

Rempel, C.B. and Hall, R. (1995). Effects of time and rate of application of triazole fungicides on incidence and severity of blackleg and growth and yield of canola. *Canadian Journal of Plant Science* **75**: 737-743.

Rimmer, S.R. and van den Berg, C.G.J. (1992). Resistance of oilseed *Brassica* spp. to blackleg caused by *Leptosphaeria maculans. Canadian Journal of Plant Pathology* **14**: 56-66.

Roderick, H.W. and Thomas, B.J. (1997). Infection of ryegrass by three rust fungi (*Puccinia coronata, P. graminis* and *P. loliina*) and some effects of temperature on the establishment of the disease and sporulation. *Plant Pathology* **46**: 751-761.

Roger, C. and Tivoli, B. (1996). Spatio-temporal development of pycnidia and perithecia and dissemination of spores of *Mycosphaerella pinodes* on pea (*Pisum sativum*). *Plant Pathology* **45**: 518-528.

Roussel, S., Nicole, M., Lopez, F., Renard, M., Chevre, A.M. and Brun, H. (1999a). Cytological investigation of resistance to *Leptosphaeria maculans* conferred to *Brassica napus* by introgressions originating from *B. juncea* or *B. nigra* B genome. *Phytopathology* **89**: 1200-1213.

Roussel, S., Nicole, M., Lopez, F., Ricci, P., Geiger, J.P., Renard, M. and Brun, H. (1999b). *Leptosphaeria maculans* and cryptogein induce similar vascular responses in tissues undergoing the hypersensitive reaction in *Brassica napus. Plant Science* **144**: 17-28.

Rouxel, T., Renard, M., Kollmann, A. and Bousquet, J.F. (1990). Brassilexin accumulation and resistance to *Leptosphaeria maculans* in *Brassica* spp. and progeny of an interspecific cross *B. juncea x B. napus. Euphytica* **46**: 175-181.

Rouxel, T., Sarniguet, A., Kollmann, A. and Bousquet, J.F. (1989). Accumulation of a phytoalexin in *Brassica* spp. in relation to a hypersensitive reaction to *Leptosphaeria maculans. Physiological and Molecular Plant Pathology* **34**: 507-517.

Roy, N.N. (1978a). A study on disease variation in the populations of an interspecific cross of *Brassica juncea* L. x *B. napus* L. *Euphytica* **27**: 145-149.

Roy, N.N. (1978b). Wesreo – A blackleg resistant rapeseed. *Journal of Agriculture, Western Australia* **19**: 42.

Roy, N.N. (1984). Interspecific transfer of *Brassica juncea*-type high blackleg resistance to *Brassica napus. Euphytica* **33**: 295-303.

Roy, N.N. and Reeves, J. (1975). Breeding better rape and linseed for Western Australia. *Journal of Agriculture, Western Australia* **16**: 93-97.

Ruzin, S.E. (1999). *Plant Microtechnique and Microscopy*. Oxford University Press, New York, USA.

Sacristan, M.D. and Gerdemann, M. (1986). Different behaviour of *Brassica juncea* and *B. carinata* as sources of *Phoma lingam* resistance in experiments of interspecific transfer to *B. napus. Plant Breeding* **97**: 304-314.

Salam, M. U., Khangura, R. K., Diggle, A. J. and Barbetti, M. J. (2002). The annual showers of blackleg ascospores in canola: Can we predict and avoid it? In: Oilseed Updates 2002. Agribusiness Crop Updates 2002, Department of Agriculture, Western Australia, South Perth, Australia pp 47-49.

Salisbury, P.A., Ballinger, D.J., Wratten, N., Plummer, K.M. and Howlett, B.J. (1995). Blackleg disease on oilseed *Brassica* in Australia – A review. *Australian Journal of Experimental Agriculture* **35**: 665-672.

Salisbury, F.B. and Ross, C.W. (1992). *Plant Physiology.* 4th edition. Wadsworth Inc., Belmont, California, USA.

Santini, A. and Capretti, P. (2000). Analysis of the Italian population of *Ceratocystis fimbriata* f.sp. *platani* using RAPD and minisatellite markers. *Plant Pathology* **49**: 461-467.

Schuerger, A.C. and Mitchell, D.J. (1993). Influence of mucilage secreted by macroconidia of *Fusarium-solani* f. sp. *phaseoli* on spore attachment to roots of *Vigna radiata* in hydroponic nutrient solution. *Phytopathology* **83**: 1162-1170.

Sexton, A.C., Kirkegaard, J.A. and Howlett, B.J. (1999). Glucosinolates in *Brassica juncea* and resistance to Australian isolates of *Leptosphaeria maculans*, the blackleg fungus. *Australasian Plant Pathology* **28**: 95-102.

Shankar, M., Cowling, W.A., and Sweetingham, M.W. (1998). Histological observations of latent infection and tissue colonization by *Diaporthe toxica* in resistant and susceptible narrow-leafed lupins. *Canadian Journal of Botany* **76**: 1305-1316.

Shaw, B. D. and Hoch, H. C. (1999). The pycnidiospore of *Phyllosticta ampelicida*: surface properties involved in substratum attachment and germination. *Mycological Research* **103**: 915-924.

Shoemaker,R.A. and Brun, H. (2001). The teleomorph of the weakly aggressive segregate of *Leptosphaeria maculans. Canadian Journal of Botany* **79**: 412-419.

Siwek, K. and Ophel-Keller, K. (1997). Calibration of a DNA-based soil assay for detection of *Rhizoctonia solani* AG-8. *Australasian Plant Pathology Society 11th Biennial Conference, Perth, Australia 1997.* p 153.

Sjödin, C. and Glimelius, K. (1988). Screening for resistance to blackleg *Phoma Lingam* (Tode ex Fr.) Desm. within *Brassicaceae. Journal of Phytopathology* **123**: 322-332.

Smith, J.E., Korsten, L. and Aveling, T.A.S. (1999). Infection process of *Colletotrichum dematium* on cowpea stems. *Mycological Research* **103**: 230-234.

Somasegaran, P and Hoben, H.J. (1985). Methods in Legume-Rhizobium technology. Nitrogen Fixation in Tropical Agricultural Legumes (NifTAL). Paia, Hawaii, USA.

Somda, I., Delourme, R., Renard, M. and Hortense, B. (1999). Pathogenicity of *Leptosphaeria maculans* isolates on a *Brassica napus-B. juncea* recombinant line. *Phytopathology* **89**: 169-175.

Somda, I., Harkous, S. and Brun, H. (1997). Bipolar heterothallism in B-group isolates of *Leptosphaeria maculans. Plant Pathology* **46**: 890-896.

Somda, I., Renard, M and Brun, H. (1996). Morphology, pathogenicity and isozyme variation amongst French isolates of *Leptosphaeria maculans* recovered from *Brassica juncea* cv. Picra. *Plant Pathology* **45**: 1090-1098.

Sosnowski, M. (1999). Interactions between canola and blackleg (*Leptosphaeria maculans*). Honours Thesis. University of Adelaide, South Australia.

Sosnowski, M., Ramsey, M., Murray, G., Scott, E. and Wilmshurst, C. (2001a). Symptoms of blackleg (*Leptosphaeria maculans*) on the roots of canola in Australia. *Plant Pathology* **50**: 808.

Sosnowski, M.R., Scott, E.S. and Ramsey, M.D. (2001b). Pathogenic variation of South Australian isolates of *Leptosphaeria maculans* and interactions with cultivars of canola (*Brassica napus*). *Australasian Plant Pathology* **30**: 45-51.

Southerton, S.G. and Deverall, B.J. (1990). Histochemical and chemical evidence for lignin accumulation during the expression of resistance to leaf rust fungi in wheat. *Physiological and Molecular Plant Pathology* **36**: 483-494.

Sreenivasaprasad, S., Mills, P.R., Meehan, B.M. and Brown, A.E. (1996). Phylogeny and systematics of 18 *Colletotrichum* species based on ribosomal DNA spacer sequences. *Genome* **39**: 499-512.

Stevenson, P.C., Turner, H.C. and Haware, M.P. (1997). Phytoalexin accumulation in the roots of chickpea (*Cicer arietinum* L) seedlings associated with resistance to fusarium wilt (*Fusarium oxysporum* f.sp. *Ciceri*). *Physiological and Molecular Plant Pathology* **50**:167-178.

Struss, D., Quiros, C.F., Plieske, J. and Robbelen, G. (1996). Construction of *Brassica* B-genome synteny groups based on chromosomes extracted from three different sources of phenotypic, isozyme and molecular markers. *Theoretical and Applied Genetics* **93**: 1026-1032.

Sun, P., Fitt, B.D.L., Gladders, P. and Welham, S.J. (2000). Relationships between phoma leaf spot and development of stem canker (*Leptosphaeria maculans*) on winter oilseed rape (*Brassica napus*) in Southern England. *Annals of Applied Biology* **137**: 113-125.

Sun, P., Fitt, B.D.L., Steed, J.M., Underwood, C.T. and West, J.S. (2001). Factors affecting development of phoma stem canker (*Leptosphaeria maculans*) on stems of winter oilseed rape (*Brassica napus*) in Southern England. *Annals of Applied Biology* **139**: 227-242.

Taylor, J.L., Borgmann, I. and Seguin-Swartz G. (1991). Electrophoretic karyotyping of *Leptosphaeria maculans* differentiates highly virulent from weakly virulent isolates. *Current Genetics* **19**: 273-275.

Terlet, J. (1992). *Scanning Electron Microscopy* – an introduction to Scanning Electron Microscopy (SEM). Centre for Electron Microscopy and Microstructure Analysis, The University of Adelaide, South Australia.

Thurling, N. and Venn, L.A. (1977). Variation in the responses of rapeseed (*Brassica napus* and *B. campestris*) cultivars to blackleg (*Leptosphaeria maculans*) infection. *Australian Journal of Experimental Agriculture and Animal Husbandry* **17**: 445-51.

Thurwachter, F., Garbe, V. and Hoppe, H.H. (1999). Ascospore discharge, leaf infestation and variations in pathogenicity as criteria to predict impact of *Leptosphaeria maculans* on oilseed rape. *Journal of Phytopathology* **147**: 215-222.

Toscano-Underwood, C., West, J.S., Fitt, B.D.L., Todd, A.D. and Jedryczka, M. (2001). Development of phoma lesions on oilseed rape leaves inoculated with ascospores of A-group or B-group *Leptosphaeria maculans* (stem canker) at different temperatures and wetness durations. *Plant Pathology* **50**: 28-41.

Tsao, P.H. and Ocana, G. (1969). Selective isolation of species of *Phytophthora* from natural soils on an improved antibiotic medium. *Nature (London)* **223:** 636-638.

Turkington, T.K., Clayton, G.W., Klein-Gebbinck, H. and Woods, D.L. (2000b). Residue decomposition and blackleg of canola: influence of tillage practices. *Canadian Journal of Plant Pathology* **22**: 150-154.

Turkington, T.K., Clayton, G.W. and Woods, D.L. (2000a). The impact of soil incorporation of canola residues and stubble application of chemicals on decomposition and inoculum production by *Leptosphaeria maculans*. *Canadian Journal of Plant Pathology* **22**: 155-159.

Tynan, K.M., Scott, E.S. and Sedgley, M. (1998). Evaluation of *Banksia* species for response to *Phytophthora* infection. *Plant Pathology* **47**: 446-455.

Vance, C.P., Kirk,T.K. and Sherwood, R.T. (1980). Lignification as a mechanism of disease resistance. *Annual Review of Phytopathology* **18**: 259-288.

van den Berg, C.G.J., Rimmer, S.R. and Parks, P. (1993). Comparison of scales for rating severity of blackleg in canola. *Canadian Journal of Plant Pathology* **15**: 49-53.

Voigt, K., Schleier, S. and Wostemeyer, J. (1998). RAPD-based molecular probes for the blackleg fungus *Leptosphaeria maculans* (*Phoma lingam)*: Evidence for pathogenicity

group-specific sequences in the fungal genomes. *Journal of Phytopathology* **146**: 567-576.

Waterhouse, G.M. and Waterston, J.M. (1966). *Phytophthora megasperma.* CMI Descriptions of Pathogenic Fungi and Bacteria. No. 115. The Eastern Press Ltd, London, UK.

West, J.S., Balesdent, M.H., Rouxel, T., Narcy, J.P., Huang, Y.J., Roux, J., Steed, J.M. Fitt, B.D.L. and Schmit, J. (2002). Colonisation of winter oilseed rape tissues by A/Tox^{+} and B/Tox^{0} *Leptosphaeria maculans* (phoma stem canker) in France and England. *Plant Pathology* **51**: 311-321.

West, J.S., Biddulph, J.E., Fitt, B.D.L. and Gladders, P. (1999). Epidemiology of *Leptosphaeria maculans* in relation to forecasting stem canker severity on winter oilseed rape in the UK [Review]. *Annals of Applied Biology* **135**: 535-546.

West, J.S., Evans, N., Liu, S., Hu, B. and Peng, L. (2000). *Leptosphaeria maculans* causing stem canker of oilseed rape in China. *Plant Pathology* **49**: 800.

West, J.S., Kharbanda, P.D., Barbetti, M.J. and Fitt, B.D.L. (2001). Epidemiology and management of *Leptosphaeria maculans* (phoma stem canker) on oilseed rape in Australia, Canada and Europe. *Plant Pathology* **50**: 10-27.

Williams, P.H. (1992). Biology of *Leptosphaeria maculans*. *Canadian Journal of Plant Pathology* **14**: 30-35.

Williams, R.H. and Fitt, B.D.L. (1999). Differentiating A and B groups of *Leptosphaeria maculans*, causal agent of stem canker (blackleg) of oilseed rape. *Plant Pathology* **48**: 161-175.

Wood, P.McR. and Barbetti, M.J (1977a). The role of seed infection in the spread of blackleg of rape in Western Australia. *Australian Journal of Experimental Agriculture and Animal Husbandry* **17**: 1040-1044.

Wood, P.McR. and Barbetti, M.J. (1977b). A study on the inoculation of rape seedlings with ascospores and pycnidiospores of the blackleg disease causal agent *Leptosphaeria maculans*. *Journal of the Australian Institute of Agricultural Science* **43**: 79-80.

Zhou, Y., Fitt, B.D.L., Welham, S.J., Evans, N. and Gladders, P. (2000). Effects of stem canker (*Leptosphaeria maculans*) and light leaf spot (*Pyrenopeziza brassicae*) on yield of winter oilseed rape (*Brassica napus*) in Southern England. *Plant Pathology* **49**: 487-497.

Zhou, Y., Fitt, B.D.L., Welham, S.J., Gladders, P., Sansford, C.E. and West, J.S. (1999). Effects of severity and timing of stem canker (*Leptosphaeria maculans*) symptoms on yield of winter oilseed rape (*Brassica napus*) in the UK. *European Journal of Plant Pathology* **105**: 715-728.

Xi, K. and Morrall, R.A.A. (1993). Histopathology of blackleg development in spring rapeseed. *Journal of Phytopathology* **137**: 26-32.

Xi, K., Morrall, R.A.A., Baker, R.J. and Verma, P.R. (1990). Relationship between incidence and severity of blackleg disease of rapeseed. *Canadian Journal of Plant Pathology* **12**: 164-169.

Xi, K., Morrall, R.A.A., Gugel, R.K. and Verma, P.R. (1991). Latent infection in relation to the epidemiology of blackleg of spring rapeseed. *Canadian Journal of Plant Pathology* **13**: 321-331.

Xue, B., Goodwin, P.H. and Annis, S.L. (1992). Pathotype identification of *Leptosphaeria maculans* with PCR and oligonucleotide primers from ribosomal internal transcribed spacer sequences. *Physiological and Molecular Plant Pathology* **41**: 179-188.